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Studies on Soybean Proteins*

Part I. Water Dispersibility of Protein of Defatted Soybean Flour as a Criterion for Degree of Denaturation**

By Danji FUKUSHIMA

Concerning the heat treatment of defatted soybean flour, the fitness of water dispersibility of protein as a criterion of the degree of denaturation has been investigated. Denaturation was measured by the change of susceptibility of the protein in water extract of the flour to protease action of *Aspergillus sojae* before and after heat treatment. It was found that the decrease of water dispersibility did not always run paralell with denaturation. The dispersibility method was applicable only to flours autoclaved for a definite period which varied according to temperature and the water content of the flour treated.

Discussion is made about the fitness of measuring the degree of denaturation by protease action.

INTRODUCTION

Shoyū (Japanese soy sauce) and miso are foods of daily necessity to the Japanese just like butter and cheese in Western countries. They have occupied a very important position in the food industries in this country. In particular, shoyū has been mass-produced on a large scale in modern industry.

Soybean and wheat are used as materials. They are autoclaved, inoculated with seed-koji (*Aspergilli*) and incubated for four days so as to grow the mold. The resulted mold koji is added with saline and incubated for approximately one year at room temperature. The soybean protein occupies about 70% of the protein in materials. The major component of protein in soybean is "glycinin". This protein is chiefly hydrolyzed by the proteases of *Aspergilli* during brewing time to give a good taste and flavour, and high nutritive value. Therefore in shoyū production it is essential to decompose the protein in materials easily and completely by the proteases in koji-mold. Since glycinin is a globular protein and the globular proteins are, in general, hardly hydrolyzed in the native state, the soybean protein must be first denatured by some procedure. Hence the denaturation of soybean proteins is a most fundamental and important subject to be investigated.

Studies on the denaturation of soybean protein have been chiefly performed on the denaturation during the oil-extracting process. With regard to its criterion the measurement of water- or salt-dispersible nitrogen has been performed. Particularly, water dispersibility has been regarded as the best criterion[1] for determining the degree of denaturation, because native soybean meal has a high dispersibility of nitrogenous constituents by water. This dispersibility is sensitively decreased by heat or practically by any other denaturing agent. The method of the water extraction has been investigated in detail by a number of workers.[2,3,4]

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** Presented at meeting of the Agricultural Chemical Society of Japan, Tokyo, September 28, 1957.

1) S. J. Circle, "Soybeans and Soybean Products, Vol. 1", Interscience Publishers, Inc., New York, 1950, p. 275.

2) A. K. Smith, S. J. Circle and G. H. Brother, *J. Am. Chem. Soc.*, **60**, 1316 (1938).

3) A. K. Smith and S. J. Circle, *Ind. Eng. Chem.* **30**, 1414 (1938).

4) R. H. Nagel, H. C. Beckel and R. T. Milner, *Cereal Chem.* **15**, 463 (1938).

It is an unusal characteristic that a very high percentage of nitrogenous matter (more than 90%) can be extracted with water from soybean meal, which differentiates soybean meal protein from other seed meal proteins. Several explanations on the high water dispersibility of soybean protein have been offered such as the potassium phosphate theory (Osborn and Campbell; 1898 and Harvath; 1937), dialyzable salt theory (Ryndin; 1936), phosphatide theory (Nagel, Beckel, and Milner; 1938), and alkalinity theory (Smith, Schubert, and Belter; 1955). However, any satisfactory explanations have not yet been obtained.

In the meanwhile, it is generally known that the degree of coagulation of proteins is greatly influenced by various factors of environment, regardless of its denaturation state. Denaturation appears to be a quite different process occurring at the different rate from that of coagulation.[5,6] Accordingly, it must be fully investigated whether or not the degree of denaturation runs paralell with the decrease of water dispersibility of soybean meal protein. However any investigation on this problem has not yet been found in the literatures. In this paper the native protein in the water extract was measured by the change of the susceptibility to the protease action of *Asp. sojae* and the fitness of water dispersibility as a criterion of the degree of denaturation was investigated.

MATERIAL AND METHODS

Preparation of Oil-Free Soybean Flour. Shirahana soybean, the variety of late-ripening, grown in 1956 in Japan were used throughout this experimental work. Material was stored in the refrigerator after it was sun-dried. Fat-free soybean was prepared by dehulling, grinding followed by the screening through a 30-mesh, then removing the oil with *n*-hexan extraction at 35°C and air-dried. The fat-free flour had been kept in refrigerator less than two months to avoid denaturation during storage. A typical fat-extracted flour sample showed the following percentage composition: moisture, 6.59; nitrogen, 8.559.

Heat Treatment of Soybean Protein. One gram of defatted soybean flour and a definite amount of water were mixed in a 25-ml Erlenmeyer flask and autoclaved.

Determination of Water-Dispersible Nitrogen. The single extraction method of Smith et al.[3] was modified as follows. Heat-treated soybean flour was transferred to a 500-ml polyethylene bottle and shaken mechanically for sixty minutes at 30°C with 200 ml water. The contents of the bottle were transferred to a 250-ml graduated flask, and the volume was adjusted to 250 ml by water. A portion was centrifuged at 2440×g for six minutes. Total nitrogen was determined on a 20 ml-sample, the supernatant from centrifugation, by the semi-micro Kjeldahl method.

Enzyme Preparation.

(1) Protease of *Asp. sojae*: The alkaline protease was isolated from *Asp. sojae* by the method of Mizunuma et al.[7]; the method included purification of the enzyme by ion exchange chromatography (IRC-50) of the water extract of a wheat bran koji. The resulted preparation contained less than 1% of original amylase activity and 2% of neutral protease, but no acidic protease.

(2) The crystalline protease of *Bac. subtilis* (Purchased from Nagase Co., Ltd.):

Determination of Protease Activity. Casein digesting activity was measured by a modification[8] of the Anson method[9]. One ml of 2% milk casein (Hammarsten) solution was poured into a test tube containing 0.5 ml of the enzyme solution and 0.5 ml of 0.2 mol. phosphate buffer (pH 7.2) and the mixture was incubated at 30°C for ten minutes. Then 2 ml of 0.4 mol. trichloroacetic acid was added to the digest to stop the reaction and the resulted precipitate was removed by filtration after standing for twenty minutes at 30°C. Five ml of 0.4 mol. sodium carbonate and 1 ml of Folin's reageut[10] were successively added to 1 ml of the filtrate and it was kept at 35°C for twenty minutes. Optical density was measured using a EPO-B Type Hitachi Photometer with a 660 mμ filter against distilled water. The same experiment was carried out with a blank which was operated essentially in the same way as above except that the enzyme was added later. Optical

5) S. Mizushima and S. Akabori, "Chemistry of Proteins, Vol. 2" Kyoritsu Press, Japan, 1954, p. 549.
6) K. J. Laidler, "Introduction to the chemistry of Enzymes", McGraw-Hill Book Company, Inc., 1954, p. 145.
7) T. Mizunuma and N. Iguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 35 (1958).
8) B. Hagihara, *Ann. Rep. Soc. Osaka Univ.*, **2**, 35 (1954).
9) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).
10) O. Folin and V. Ciocalten, *J. Biol. Chem.*, **73**, 627 (1927).

density of the blank test was subtracted from that of the test run and converted into mg equivalent tyrosine liberated by enzyme action. One unit, $[PU]^{cas.\ 30'\ FR}_{m.\ eq.\ tyr.}$, represented the activity which liberated 1 mg equivalent of tyrosine in trichloroacetic acid-soluble fraction per minute under the above conditions.

Enzymatic Hydrolysis of Water-Extracted Soybean Protein. A mixture of 1 ml of substrate solution, 1 ml of enzyme solution, and 0.5 ml of 0.2 mol. phosphate buffer solution (pH 7.2) was incubated at 30°C for a definite time. One ml of 1.2 mol. trichloroacetic acid was added to stop the reaction and the filtration was done after complete formation of the precipitate. One ml of the filtrate was assayed for protein as described above. The value was expressed as the correspondent amount of tyrosine (γ) which represented the degree of hydrolysis.

RESULTS

I. Water Dispersibility of Soybean Flour Protein

(1) Effect of Time and Temperature at Autoclaving: To examine whether water dispersibility is adequate as a criterion of the degree of denaturation of soybean flour protein, the effect of time and temperature in autoclaving was investigated. Fig. 1 shows the effect of time and temperature in autoclaving on water dispersibility with soybean flour to which an equal amount of water was added. At 100°C, the extracted nitrogen gradually decreased up to one hundred and twenty minutes, when dispersibility reached its minimum. At 110°C or 120°C, minimum dispersibility occurred after a few minutes and afterwards extracted nitrogen increased. The higher the temperature, the larger was the increase of the dispersed nitrogen. These results showed the unfitness of water dispersibility as a criterion of denaturation because water dispersibility of the soybean protein increased with heating time after its minimum point was attained.

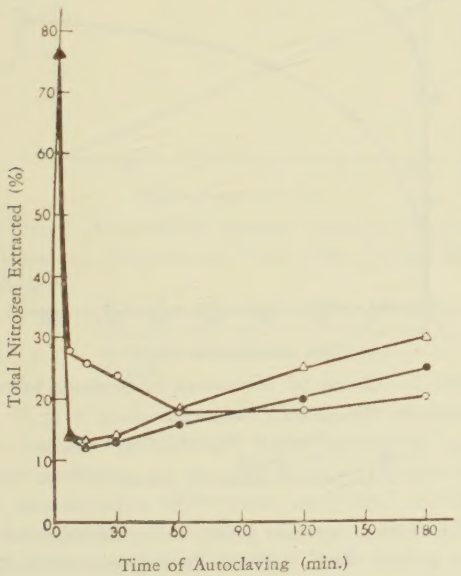

FIG. 1. Effect of Time and Temperature on Water Dispersibility of Soybean Flour Protein Autoclaved under Addition of 100% Water.
—○— 100°C, —●— 110°C, —△— 120°C

(2) Effect of Water Content: The water content of flour before autoclaving had great influence on water dispersibility as shown in Figs. 2 and 3. Fig. 2 shows

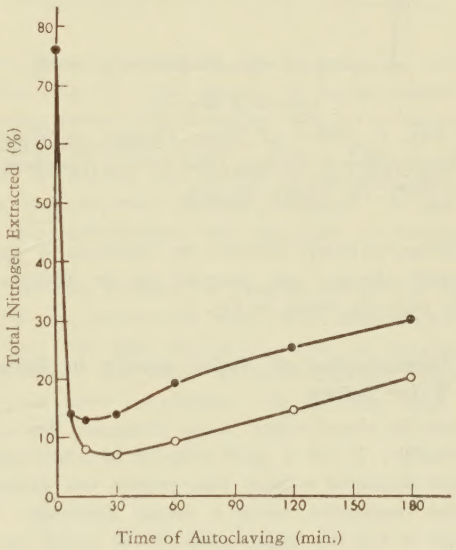

FIG. 2. Effect of Time and Water Content on Water Dispersibility of Soybean Flour Protein Autoclaved at 120°C.
—○— Without addition of water
—●— With addition of water

the water-dispersible nitrogen of the flour autoclaved at 120°C with and without the addition of an equal amount of water. The amount of water-dispersed nitrogen was larger in the flour added with water than in flour without water addition. In fact, denaturation occurred more easily in the former than in the latter, as described later.

After autoclaving the samples containing different levels of water at a definite temperature (100°C) for

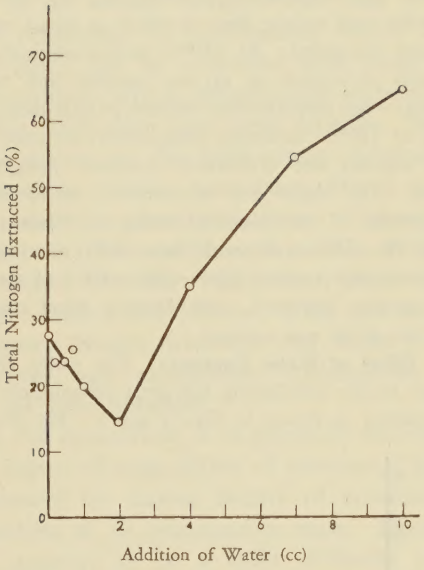

FIG. 3. Effect of Water Content on Water Dispersibility of Soybean Flour Protein Autoclaved at 100°C for Thirty Minutes.

definite period (thirty minutes), the minimum of water-dispersed nitrogen was observed by the addition of 200% water as shown in Fig. 3.

II. Determination of Native protein in Soybean Water Extract

From the above results, it was recognized that water dispersibility is not a good criterion of denaturation, because denatured soybean flour protein was extracted without coagulation under a certain condition. On account of this the measurement of the native protein in water extract by protease action was examined.

The water extract of native flour was divided into two parts: the one was added with the proteases without heating the flour and the other was heated at 100°C for ten minutes before protease addition. The results are shown in Figs. 4 and 5. As shown the protein of the heated aqueous extract was digested much faster than that of the unheated one. In this case no precipitates were produced in the water extract of flour, as long as it was diluted below definite concentrations. Moreover, there was no substance inhibitory to protease action of *Aspergillus sojae* or *Bacillus subtilis* in the extract (Table I). Accordingly, it was reasonable to attribute this increased susceptibility of protein in the heated

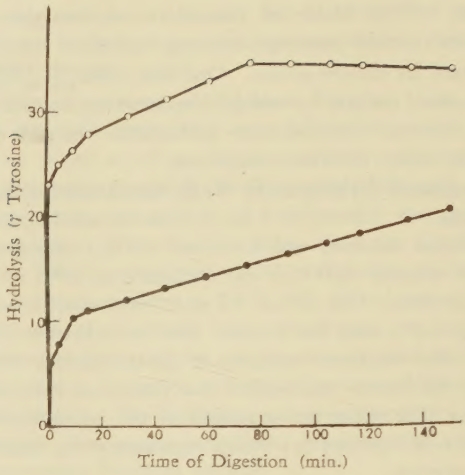

FIG. 4. Course of Hydrolysis of Soybean Water Extract by *Asp. Sojae* Protease.
—○— Treated at 100°C for fifteen min.
—●— Not treated
Total nitrogen of substrate: 0.48 mg/cc

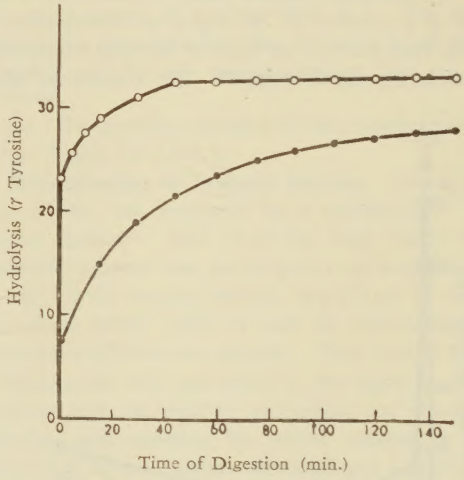

FIG. 5. Course of Hydrolysis of Soybean Water Extract by *Bac. Subtilis* Protease.
—○— Treated at 100°C for fifteen min.
—●— Not treated
Total nitrogen of substrate: 0.48 mg/cc

water extract to protease action to modification of the native protein itself. Thus, the following techniques were set up for determining the native protein in solution. If necessary, the solution was diluted, and then it was divided into two parts, one being unheated and the other heated at 100°C for ten minutes. Each part

TABLE I. EFFECT OF SOYBEAN WATER EXTRACT ON PROTEASE ACTION

Method: Experimental conditions were the same as "Determination of Protease Activity" in the present paper. The value was expressed in γ tyrosine.

	Protease of *Asp. Sojae*		Protease of *Bac. Subtiles*
	Refined Alkaline Protease	Water Extract of Wheat Bran Koji	Crystalline Protease
Control	24.8	23.8	33.1
(Control)+(Soybean Water Extract)	23.1	23.0	32.0

was added simultaneously with the alkaline protease of *Asp. sojae* (total activity; $1.0\times10^{-3}[\mathrm{PU}]^{\text{cas. 30° FR}}_{\text{m. eq. tyr.}}$), and incubated for thirty minutes under the same conditions described in "Materials and Methods". The difference of value between the two parts represented the amount of native protein in solution. If measurement was made in samples of the definite concentrations of protein, this difference was proportional to the amount of the native protein in the sample as shown in Fig. 6.

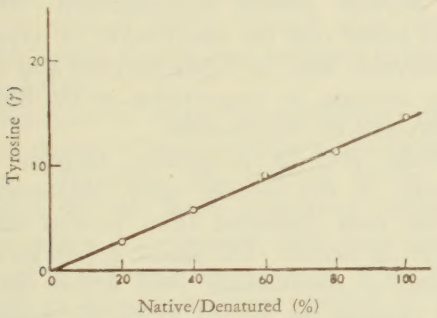

FIG. 6. Relationship between Amount of Native Protein in Solution and Value Obtained by this Method.

Substrate: Total nitrogen 0.4 mg/cc. The ratio of the unheated water extract to the heated (100°C, 10 min.) water extract was varied as indicated.

Figs. 7, 8, 9 shows the amount of native protein* in water extracts of soybean flours autoclaved under various conditions, by the application of this method.

By autoclaving at 100°C under the addition of 100% water to the flour, the amount of native protein in the water extract gradually decreased with heating time and reached zero after one hundred and twenty minutes, whereas at 110°C and 120°C, the native protein in the

* Strictly this value is not quantitative, because the protein concentration of the substrates was not regulated.

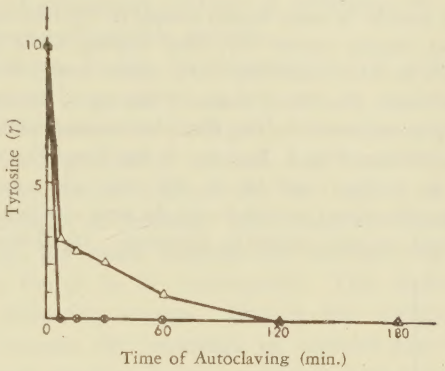

FIG. 7. Native Protein in Water Extract of Soybean Flour Autoclaved under Addition of 100% Water.

—△— 100°C —○— 110°C —×— 120°C

water extract disappeared after heating for several minutes (Fig. 7). The amount of native protein in the water extract always reached zero at the minimum of water-dispersible nitrogen (Fig. 1, Fig. 7). The longer the heating time beyond the minimum was, the more total dispersed nitrogen amounted. A part of this increased-nitrogen was originated in denatured protein.

It was very important to examine the effect of water content on denaturation and water dispersibility of proteins. The flour autoclaved under the addition of 100% water was larger in water-dispersed nitrogen than that under no addition. On the other hand, the

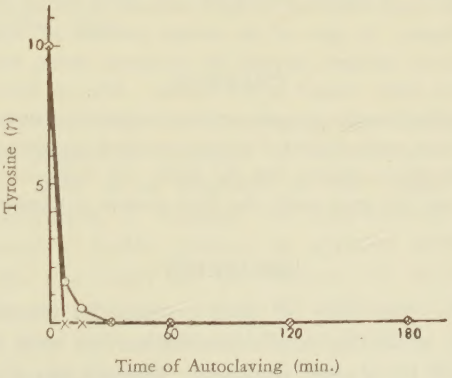

FIG. 8. Native Protein in Water Extract of Soybean Flour Autoclaved at 120°C.

—○— Without addition of water
—×— With addition of 100% water

native protein in water extract assayed by the enzymatic method already reached zero after heating for a few minutes in flour containing 100% water, while it required more than thirty minute's heating to be completely denaturated in the flour heat-treated without water addition (Fig. 8, Fig. 2). In Fig. 2 and Fig. 8 as well as in Fig. 1 and Fig. 7, the time when native protein disappeared coincided with the point at which the dispersed nitrogen reached its minimum. Fig. 9 showes

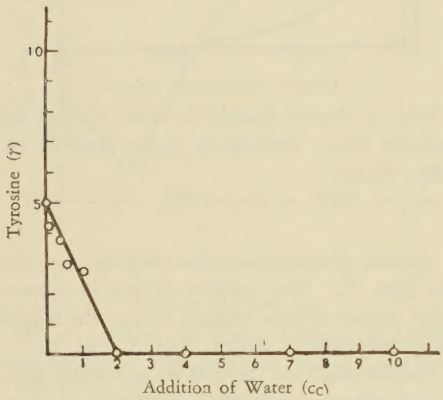

FIG. 9. Native Protein in Water Extract of Soybean Flour Autoclaved at 100°C for Thirty Minutes.

the effect of water addition to flour prior to autoclaving on the amount of native protein contained in the water extract. By autoclaving the flour contained twice as much water at 100°C for thirty minutes, the resulted water extract contained no native protein ; the minimum of the water-dispersed nitrogen occurred under the same conditions. In spite of the abrupt increase of water-extracted nitrogen beyond the minimum point, native protein never existed in the extract. Also, in this case the native protein in water extract completely disappeared when water-dispersed nitrogen reached its minimum. It therefore appears that the higher the water content became, the more easily the flour protein is denatured.

DISCUSSION

The possibility of using protease for measurement of the degree of denaturation has been suggested by several workers. Linderstrøm-Lang et al.[11] presented data indicating that native lactoglobulin might not be entirely attacked by enzyme and Haurowitz et al.[12] have pointed out that the susceptibility of native fibrous proteins to the action of trypsin is attributed to the expanded configulation of their peptide chains rendering their peptide bonds accessible to the enzyme. Lineweaver et al.[13] and Rice et al.[14] tried to measure the degree of denaturation of human serum albumin or hemoglobin by means of papain susceptibility. Bernheim et al.[15] carried out measurements of the rates of tryptic hydrolysis of horse serum albumin and horse pseudoglobulin in an attempt to detect differences in the internal structure of some proteins, i.e., native, irreversibly denatured, and the so-called regenerated proteins. Recently, Okunuki et al.[16,17] have described a quantitative method for the determination of the degree of denaturation of enzyme proteins, using crystalline protease of *Bac. subtilis* which did not act on the native protein of enzymes and hydrolyzed the denatured enzyme protein quantitatively. These authors found that the inactivation of crystalline enzymes, such as Taka-amylase and *Bac. subtilis* amylase, is proportional to the degree of hydrolysis by the protease.

Globular proteins, in general, increase susceptibility to the protease after denaturation. This has been explained from various observations[18] as follows. The atomic groups serving as points of combination with active centers of the protease are located inside the molecule in native globular proteins and are inaccessible to the protease. Denaturation which apparently involves an unfolding of the closely packed

11) K. Linderstrøm-Lang, R. D. Hotchkiss and G. Johansen, *Nature*, **142**, 966 (1938).
12) F. Haurowitz, M. Tunca, P. Schwerin and V. Goksn, *J. Biol. Chem.*, **157**, 621 (1945).
13) H. Lineweaver and S. R. Hoover, *J. Biol. Chem.*, **137**, 325 (1941).
14) R. G. Rice, G. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lumi, *J. Biol. Chem.*, **158**, 609 (1946).
15) F. Bernheim, H. Neurath and J. Q. Erickson, *J. Biol. Chem.*, **144**, 259 (1942).
16) K. Okunuki, B. Hagihara, H. Matsubara, T. Nakayama and M. Yanagida, *Symposia on Enzyme Chemistry (Japan)*, **9**, 1 (1954).
17) K. Okunuki, B. Hagihara, H. Matsubara and T. Nakayama, *J. Biochem. (Japan)*, **43**, 453 (1956).
18) F. Haurowitz, "Chemistry and Biology of Proteins", Academic Press, New York, 1950, p. 127.

peptide chains, renders these groups accessible to the protease. Therefore, the value of the increase of susceptibility of the protein to the protease should be considered to be that directly reflecting the expanded configulation of the molecule. The major proteins of soybean are of the globular type and increase susceptibility to protease as shown in Figs. 4 and 5. Therefore, the measurement of the native protein in soybean meals by using protease should be regarded to be the most suitable method.

The proteases suitable for the measurement of native protein in water extract of soybean meal or flour should have its optimum pH around the neutral pH and not be inhibited by any constituents in the water extract. It was found that both protease of *Aspergills sojae* and *Bacillus subtilis* are suitable for the above conditions (Fig. 3, Fig. 4). Nevertheless, protease of *Aspergills sojae* was used in this paper because protease of *Aspergills sojae* had less activity toward native protein than that of *Bacills subtilis* though both proteases hydrolyzed it gradually.

Inadequacy of water dispersibility as criterion of the degree of denaturation is shown in this paper. However, this method serves as a criterion for the flours autoclaved within the range where the minimum point is not reached, because in this range the amounts of the native protein in water extract run paralell to water dispersibility. It should be mentioned, however, that even within the above range the absolute amount of nitrogen dispersed by water after heat treatment varies significantly depending upon the temperature or water contents of flour. And, with regard to the samples which are treated at different conditions of temperature and water content of flour, the dispersed nitrogen does not always run paralell to the amount of the native protein. For this reason, the dispersibility method is not satisfactory for comparison of the degree of denaturation of these samples. That the denatured soybean protein is solubilized by water content, may be explained in connection to the fact[19] that the coagulation of denatured proteins is inhibited by the existance of soluble protein.

Hitherto studies for denaturation of soybean proteins have been carried out in regard to heat denaturation during oil extraction and the water dispersibility method has been used as the only useful criterion for the degree of denaturation of soybean meal protein. Further, so far, the water dispersibility method has not been found to be inadequate. This is due to the following reason. During the oil-extracting process the treatment of samples has been carried out at a lower temperature and with a lower water content of flour. On account of this the measurement of denaturation resulted has only been performed upon samples which have not attained a minimum point of water dispersibility. Thus, water dispersibility method has been used as a criterion of denaturation without considerable contradiction being recognised.

Since the protein in native soybean flour is very soluble in water, the native protein in heat-treated flour is expected to be solubilized easily and completely by water. On account of this, the protease method described here is usuable for measurement of the total amount of the native protein contained in soybean flour itself. Whether or not the native protein in heated flour is extracted as easily and completely as that in unheated protein will be described later.

SUMMARY

(1) In order to investigate the fitness of water-dispersible nitrogen of heat-treated soybean flour as a criterion of the degree of denaturation, a method for determining the amount of native protein in aqueous solution which was based on the increase of susceptibility to protease action of *Asp. sojae* prior and after heat treatment was established.

(2) In the autoclaving of flour, there existed a minimum of water-dispersed nitrogen with

19) S. Mizushima and S. Akabori, "Chemistry of Proteins Vol. 2", Kyoritsu Press, Japan, p. 550.

regard to the heating time or the water content of flour. The higher the temperature of treatment or the water content of flour, the larger was the increase of dispersed nitrogen beyond the minimum.

(3) The higher the temperature or the water content of flour, the easier denaturation occurred. The native protein in the water extract of heated flour disappeared at the minimum point of water dispersibility.

(4) The water dispersibility method served a criterion of denaturation for the flour heated within the range where a minimum point of water dispersibility was attained. Within this range amounts of water-dispersed nitrogen ran paralell to the amount of the native protein content. However, this water dispersibility method is not suitable for comparison of the degree of denaturation of flours treated under different conditions.

Acknowledgement. The author wishes to acknowledge his indebtness to Prof. K. Sakaguchi and Prof. R. Sasaki of Uhiversity of Tokyo for their kind guidance and encouragement throughout this work, and also to Assist. Prof. H. Takahashi, University of Tokyo, and Dr. F. Yoshida of this Institute for their valuable suggestions. He also wishes to express sincere thanks to Dr. M. Mogi, President of this Institute, for his support and encouragement.

Studies on Soybean Proteins*

Part II. A New Method for Quantitative Determination of the Degree of Denaturation of Protein in Soybean Flour**

By Danji Fukushima

Owing to the inadequacy of the water dispersibility method as a criterion for denaturation of the protein in soybean flours, a new method was devised which was quantitative and applicable to soybean flours denatured under various conditions. In this method the native protein in flour is determined by the quantity of protein which is solubilized but remains unhydrolyzed by the protease action of *Asp. sojae*. Conditions for the determination were decided as the result of various experiments.

It was found that the decrease in the amount of water-extractable protein in heated flour with low water contents was attributable to the prevention of extraction of native protein by partly denatured protein.

INTRODUCTION

Beck et al.[1] had expressed the degree of denaturation by heat and moisture quantitatively, using water dispersibility of the nitrogen compounds of soybean oil meal as a criterion. However, this expression was inadequate and not universal, because it was shown in the previous report that water dispersibility is applicable as a criterion only upon meals denatured under extremely limited conditions and also that it was not quantitative.

A satisfactory method for determining the degree of denaturation of soybean meal has so far been reported. The author previously reported a method for determining native protein in water extract of soybean meal by protease action, but has not investigated whether or not the degree of denaturation of the flour is indicated by the amounts of the native protein contained in the water extract. In the present paper, this point was investigated precisely and a new method was proposed for the determination of denaturation of soybean. This method is quantitative for determining the amounts of native protein in a variety of heat-treated flours.

METHOD

Sample Preparation The defatted soybean flour which was used in this paper was the same as previously reported. It showed the following percentage composition: moisture, 7.01; nitrogen, 8.045. Heat treatment was also carried out by the same procedure. Heating of flours without the addition of water was carried out in the atmosphere at a definite temperature.

Protease Solution and Reagents

Protease Solution: The water extract of wheat bran koji of *Asp. sojae* KS was diluted by distilled water so that 10 ml of the diluted solution had an activity of $6.5\times10^{-3}[\mathrm{PU}]^{\text{cas. }30^{\circ}\text{ FR}}_{\text{m. eq. tyr.}}$ at pH 7.2. The method of measurement of enzymatic activity has previously been described.

Reagents:

Buffer Solution .....One half and 0.25 mol. solution of phosphate buffers (pH 7.2)

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** Presented at the meeting of the Agricultural Chemical Society, Tokyo, September, 28, 1957.

1) A. C. Beckel, W. C. Bull and T. H. Hopper, *Ind. Eng. Chem.*, **34**, 973 (1942).

was prepared.
Trichloroacetic Acid...A 2.4 mol. solution was used.
Gum arabic .....A two per cent solution was prepared.
Toluol.....This was used as preservative.

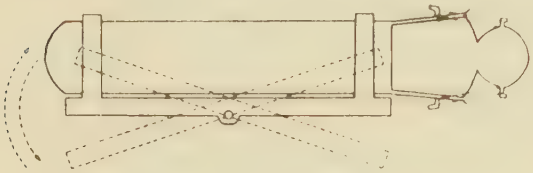

FIG. 1. Apparatus of Digestion.

Apparatus For digestion by the protease, the apparatus shown in Fig. 1 was used. The tube had a capacity of about 25 ml. In this tube, the sample was put in, the enzyme solution and other reagents were added and it was stoppered tightly with grease and rubber strips. Then the mixture was placed in a water bath at 30°C and shaken at the rate of 60 r.p.m.

Procedure The flour sample, corresponding to 1 g of flour before heat treatment, was transferred with 10 ml of 0.5 mol. phosphate buffer into the tube. Ten ml of the enzyme solution and 1 ml of toluol were added successively. After being stoppered, they were shaken for twenty hours at 30°C, as described above. At the end of this period the digestion mixtures were transferred to a graduated flask and made up to 50 ml with 0.25 mol. phosphate buffer. The resulted mixture was centrifuged for twenty minutes at 12,000 r.p.m. One ml of each supernatant was trasferred into two test tubes (A and B). To tube A, 6 ml of distilled water, 2 ml of 2% gum arabic solution, and 1 ml of 2.4 mol. trichloroacetic acid were added successively and mixed. To tube B, 9 ml of 0.25 mol. phosphate buffer was added and mixed. Tube A was incubated for twenty minutes at 30°C, after which it was shaken vigorously. Three minutes later the absorption of the solution in tube A was read against that in tude B using a Hitachi EPO-B photometer with a 660 mμ filter. Shaking of the tube was necessary to obtain reproducible data, otherwise the substrate which was solid, has to be kept in a locally-concentrated state during digestion time.

EXPERIMENTAL

I. Extraction of Native Protein by Protease

If the native protein in the flour was completely extracted by water, regardless of the conditions of denaturation of the flour, the amounts of the native protein in water extract served as a criterion for the degree of denaturation of the flour. The method reported previously, by which the native protein in the aqueous solution could be measured would be applicable for the flour itself as well as for its water extract. The protease solution of *Asp. sojae* was added to the flours which had been autoclaved for various periods at 120°C without being added water. It was found that large amounts of protein were solubilized but not hydrolyzed in the solution. The amounts of the protein solubilized but not hydrolyzed after twenty hour's digestion at 30°C were measured by the turbidity method as shown in Fig. 2. As shown in Fig. 3, the amount

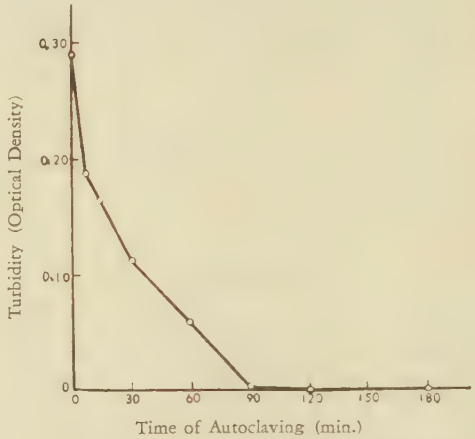

FIG. 2. Extraction of Protein in Soybean Flour Autoclaved at 120°C without Addition of Water by Protease Action.
The condition of digestion is the same as "Method".

of nitrogen compounds extracted from the flours heat-treated under these conditions by 1/4 mol. phosphate buffer was very little. This could not be attributed to the effect of salt solution, because water extraction gave almost the same extent of solubilisation. Thorefore, it appeared that there existed a protein which could not be extracted without the addition of protease.

The following experiment was performed to ascertain whether the solubilized but unhydrolyzed protein was native. For this purpose, the method reported previously in which the amounts of the native protein in the solution were determined was modified and applied to the solution of the digestion mixture, as described below.

The digestion mixtures were centrifuged and the supernatant were divided into two parts, one which was not heated and the other heated at 100°C for ten minutes. One ml of each portion was pipetted into a test tube. One ml of the solution of alkaline protease prepared from the culture medium of *Asp. sojae* by the method of Mizunuma et al.[2] was added to each tube. Initial activities of the protease solution added to each had been regulated beforehand so that both digestion mixtures had an activity of $1.0 \times 10^{-3}[PU]^{cas.\ 30'\ FR}_{m.\ eq.\ tyr.}$. After thirty minute's digestion at 30°C the amount of the trichloroacetic acid-insoluble fraction was measured by the turbidity method. The difference between the two indicated the amounts of the native protein in the solution. The results are shown in Table I. On the other hand there existed only little or no native protein which was extracted by water (Table III). In spite of the fact that there was no native protein in the flour autoclaved for thirty minutes under these conditions, large amounts of native protein were extracted by protease action. Accordingly, the measurement of the denaturation degree of the flour by estimating the native protein in the water extract was meaningless. Therefore the native protein in the flour should first be solubilized by protease action. If adequate conditions for the protease action were chosen, the native protein could be measured. The following examination was performed to find out favorable conditions.

TABLE I. NATIVE PROTEIN EXTRACTED BY PROTEASE ACTION FROM SOYBEAN FLOUR AUTOCLAVED AT 120°C WITHOUT ADDITION OF WATER

Time of Autoclaving (min.)	Protein Extracted by Protease Action (optical density)	Difference (Amount of Native Protein) (optical density)
15	0.169	0.073*
30	0.120	0.065
60	0.055	0.024

* The true value is smaller than this, because of occurrence of precipitation by heat treatment.

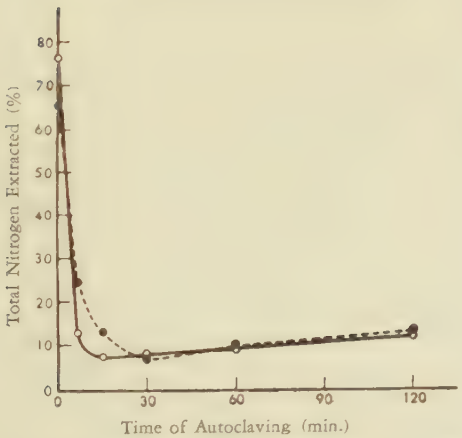

FIG. 3. Water- or Salt-Dispersibility of Soybean Flour Protein Autoclaved at 120°C without Addition of Water.
—○— Water
—●— 1/4 mol. phosphate buffer (pH 7.2)

In this article, "native protein" indicates the protein which showed an increased susceptibility to protease action after heat treatment in an aqueous state though hardly hydrolyzed before the treatment.

II. Digestion Time

In view of the susceptibility of the soybean flour protein to the protease action, two cases should be considered. The one is the increase of the susceptibility by heat treatment and the other the decrease. The former is the "denaturation" described previously in which denatured protein was usually insoluble in water. However, when the soybean flour was heated under particular conditions, the denatured protein was partly soluble in water. On the other hand, a decreased susceptibility was observed upon the flour autoclaved at a high temperature for a long period. This phenomenon was usually explained by the Melanoidin reaction that is the reaction between proteins and sugars to form complexes. The complexes were, however, considered to be hardly soluble in water. In the present method the "native protein" which was solubilized but remained unhydrolyzed by protease action, was measured. Therefore, the protein which was denatured and insolubilized after heat treatment did not have affect to this method. The denatured but solubilized protein which could be activated by the enzyme, had influence on determination of the native protein by this method. Besides these, native but insolubilized protein described in paragraph I, also had influence on this method. If digestion time was too short, there was a possibility that the native but insolubilized protein is not completely extracted, as well as the possibility of the denatured but solubilized protein being left in the solution without being hydrolyzed completely. On the other hand, if digestion was extended for a period too long, the accuracy of measurement lowered, because the native protein was hydrolyzed gradually,

2) T. Mizunuma and N. Iguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 35 (1958).

as had been shown in the previous report.

From these considerations, the duration of digestion was examined and decided to be twenty hours. This period of digestion was quite reasonable as shown in the following three experiments. Firstly, the flours autoclaved under conditions where the denatured but solubilized protein was produced in large quantities, were digested. The denatured but solubilized protein was hydrolyzed completely during this period (Table IV). Secondary, the flours autoclaved under the condition (at 120°C without added water) where the native but insolubilized protein is produced in large quantities, were digested for twenty hours. The resulted mixtures were centrifuged off and the precipitates were digested once more. Any protein was not solubilized from the precipitates thus showing that the extraction of the native but insolubilized protein is completed during the above digestion period. Finally, as shown in Fig. 4, when heat-treated flour was used

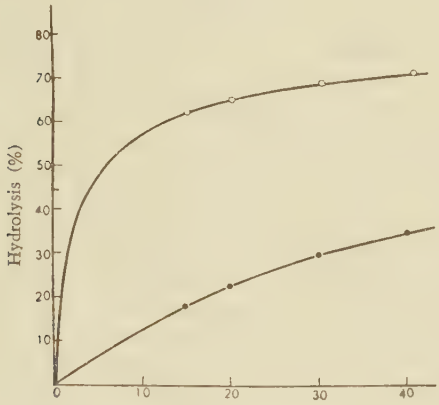

FIG. 4. Hydrolysis of Soybean Flour Protein by Protease of *Asp. sojae*.

—○— Treated at 100°C for 30 min. under the addition of 5-folds of water

—●— Not treated

Enzyme and Conditions of Digestion: The same as the "Method" in this paper.

Degree of Hydrolysis: Trichloroacetic acid-soluble nitrogen was measured.

as the sample, approximately 70% of the total protein of the flour was hydrolyzed after twenty hour's digestion time. When native flour was used, only about 20% of the total protein was hydrolyzed under the same conditions. From the above three experiments, it was decided that a digestion of twenty hours was most suitable for this method.

III. Examination of Quantitative Relationship

(1) Relationship between Turbidity and Protein Concentration: In order to obtain quantitative results the turbidity appearing after the addition of trichloroacetic acid to the protein solution should be proportional to the protein concentration. The proportional relationship was obtained only when gum arabic was not put into a blank (Fig. 5). Although the gum arabic solution was somewhat opaque, the error resulting from the omittion of gum arabic to the blank was almost negligible in this measurement.

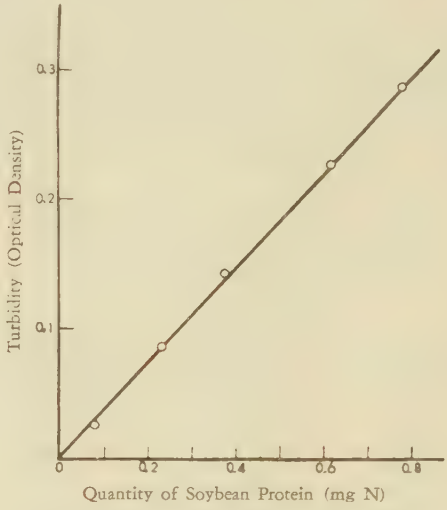

FIG. 5. Relationship between Turbidity and Soybean Protein Concentration.

(2) Relationship between Turbidity and Native Protein Quantity in Flour: It was subsequently examined whether or not the turbidity method applied to the flour was proportional to the native protein content of the flour. At the same time the effect of the digestion period was also examined. The native flour and the completely denatured flour were mixed with various ratios and 1 g of each of these mixtures was digested for five, twenty, and forty hours, respectively. As shown in Fig. 6, the relationship between turbidity and protein content was essentially linear, regardless of the length of digestion time.

IV. Determination of Degree of Denaturation of Protein in Heat-Treated Soybean Flour

The amounts of the native protein in the heat-treated flours were determined by this method (Tables II, III, and IV). The water-dispersed nitrogen of these flours

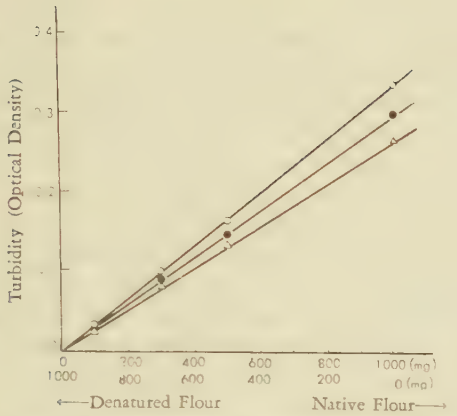

FIG. 6. Relationship between Turbidity and Native Protein Quantity in Soybean Flour.
—○— Five hours in digestion time
—●— Twenty hours
—△— Forty hours

TABLE II. WATER-DISPERSED NITROGEN, WATER-EXTRACTED NATIVE PROTEIN, AND TOTAL NATIVE PROTEIN IN FLOUR AUTOCLAVED UNDER ADDITION OF 100 PERCENT WATER

Temperature of Autoclaving	Time of Autoclaving (min.)	Water-dispersed Nitrogen (%)	Water-extracted Native Protein (γ Tyrosine)	Total Native Protein in Flour (Turbidity)
	Native	74.4	9.6	0.301
100°C	7	28.2	2.9	0.146
	15	27.0	2.7	0.137
	30	24.1	2.5	0.114
	60	18.6	1.0	0.080
	120	18.6	0	0.023
	180	19.6	0	0.002
110°C	7	13.6	0	0
	15	12.7	0	0
	30	13.7	0	0
	60	17.0	0	0
	120	20.1	0	0
	180	24.5	0	0
120°C	7	13.9	0	0
	15	13.7	0	0
	30	14.7	0	0
	60	19.4	0	0
	120	24.4	0	0
	180	29.8	0	0

TABLE III. WATER-DISPERSED NITROGEN, WATER-EXTRACTED NATIVE PROTEIN, AND TOTAL NATIVE PROTEIN IN FLOUR HEATED AT 120 DEGREE CENTIGRADE

Water Content of Flour before Heating and Heating Procedure	Time of Heating (min.)	Water-dispersed Nitrogen (%)	Water-extracted Native Protein (γ Tyrosine)	Total Native Protein in Flour (Turbidity)
	Native	77.0	9.7	0.302
P_2O_5-Dehydrated and Dry Heating	60	72.6	—	0.281
	120	66.4	—	0.275
	180	60.8	—	0.252
	240	46.7	—	0.240
	300	34.9	—	0.229
Non Water-Added and Autoclaving	7	14.9	1.4	0.186
	15	7.7	0.5	0.166
	30	7.4	0	0.104
	60	9.1	0	0.060
	90	12.0	0	0.005
	120	14.5	0	0
	180	20.1	0	0
100% Water-added and Autoclaving	7	13.9	0	0
	15	13.7	0	0
	30	14.7	0	0
	60	19.4	0	0
	120	24.4	0	0
	180	29.8	0	0

and the native protein in their water extracts is also shown in the Tables.

By autoclaving at 100°C under a 100%-addition of water, a small amount of native protein still remained in the flour after three hour's heating. However, the native protein completely disappeared after a few minutes heating at 110°C and 120°C (Table II). On the other hand the native protein in the water extract disappeared after heating for two hours at 100°C (Table II). It was observed that an insolubilization of the native protein not being due to denaturation slightly occurred by heat treatment at 100°C under 100%-addition of water.

When the flour was autoclaved without any addition of water, the denaturation did not easily occur even at 120°C. And, the native protein in the flour did not disappear, until the flour was heated for ninety minutes (Table III). On the other hand, the native protein in the water extract disappeared, by heating for thirty minutes. At this heat-time, 35% of the

TABLE IV. WATER-DISPERSED NITROGEN, WATER-EXTRACTED NATIVE PROTEIN, AND TOTAL NATIVE PROTEIN IN FLOUR AUTOCLAVED AT 100 DEGREE CENTIGRADE FOR THIRTY MINUTES

Amount of Water Added to Flour as Autoclaved (cc)	Water-Dispersed Nitrogen (%)	Water-Extracted Native Protein (γ Tyrosine)	Total Native Protein in Flour (Turbidity)
Native	74.4	9.6	0.301
0	27.9	4.9	0.186
0.1	23.9	4.1	0.173
0.3	23.6	3.8	0.166
0.5	25.3	3.0	0.160
1.0	20.0	2.8	0.100
2.0	15.6	0	0.004
4.0	34.5	0	0
7.0	57.3	0	0
10.0	64.3	0	0

initial native protein in fact, was remaining in the flour. This indicated that the insolubilization of the native protein occurred in a greater extent under these conditions. The water dispersibility of the nitrogen of this flour was lower than that of the flour autoclaved at the same temperature with a 100%-addition of water. Therefore, the water dispersibility did not serve as a criterion for the degree of denaturation.

The flour which had been dehydrated for several days at room temperature in a vaccum desicator containing P_2O_5, was heat-treated at 120°C without the addition of water. It was found that both the native protein and the water-dispersed nitrogen decreased together with heat time. But the decrease of water-dispersed nitrogen was more remarkable than that of the native protein (Table III). Therefore the native but insolubilized protein tended to increase under a long dry heating.

By autoclaving the flour with definite amounts of water at 100°C for thirty minutes, the native protein in both the flour and its water extract disappeared completely at a point where the extracted nitrogen reached its minimum level: this occurred in the flour which contained a 200%-addition of water (Table IV). Presumably, the insolubilization of the native protein which was not due to denaturation did not occur in the presence of 200% of water.

DISCUSSION

This method determined a protein which was soluble in water and hardly hydrolyzed by the protease action of *Asp. sojae*. Soybean flour consisted of various components of the protein. Some of them were insoluble in water and some were easily hydrolyzed by protease action in the native state. Therefore, the absolute degree of denaturation could not be measured by this method, because soybean flour contained various proteins. However, more than 60%* of the total proteins in soybean flour were water-soluble proteins, whose susceptibilities to protease action remarkably increased upon heat treatment. Accordingly, the degree of denaturation of soybean proteins could be represented by those proteins.

Of particular significance was the discovery that the native protein was not extracted by either water or buffer solution (Fig. 3), when the protein in the flour was partially denatured under low water contents. It was not examined whether or not this native but insolubilized protein was partly modified, though the susceptibility to the protease action after treatment could not be distinguished from unheated protein. A most probable explanation for this phenomenon might be that the heat-treated flour particles with a low water content, covered by the denatured and insolubilized protein, and the remaining native protein constituted a core of its particle. Thus, the particle seemed to behave insoluble before protease action, by which the insoluble membrane of the particles was solubilized by the enzyme, and the core-native protein came out into the solution. The observation on the appearance of trichloroacetic acid-soluble nitrogen compound prior to that of the native protein during the digestion of particles was indicative. Of course, other possibility could not be excluded. According to such a phenomenon, the water dispersibility method should not be applied to flour, which were heat-treated with low water contents. In the meanwhile, it had been discussed in the previous report that the method based on protease action directly indicated a change of the

* This value was calculated from the amounts of protein measured by turbidity in this method.

configulation of protein molecules, differing from the procedures by the dispersibility of protein in water, salts, or alkaline solution. Therefore the method described here should be regarded to be most suitable for measuring the protein in soybean flour itself.

SUMMARY

(1) The majority of soybean proteins where hardly hydrolyzed in native state by the protease of *Asp. sojae*, but became susceptible to the enzyme upon heating in a solubilized state. From this fact the method for determining the native protein in the soybean flour was investigated.

(2) The insolubilization of the protein, which occurred by heating under low water contents, was not attributed to its denaturation. The native protein in these flour was not extracted by water or buffer solution, but could be solubilized after the protease action. Therefore the measurement of the native protein in the water extract by the method previously described was inadequate for the purpose of measuring a degree of denaturation of heat treated flours.

(3) A new method was described, which was quantitative and applicable to the flours heated under various conditions. In this method the native protein in the flours was determined by applying the turbidimetric method to proteins which were extracted but unhydrolyzed after a twenty-hour digestion (30°C, pH 7.2) of flours by the protease of *Asp. sojae* with an activity of $6.5\times10^{3}[\mathrm{PU}]^{\text{cas. }30^{\circ}\mathrm{FR}}_{\text{m. eq. tyr.}}$ at pH 7.2.

Acknowledgement. The author wishes to acknowledgement his indebtness to Prof. K. Sakaguchi and Prof. R. Sasaki of University of Tokyo for their kind guidance and encouragement throughout this work, and also to Assist. Prof. H. Takahashi of University of Tokyo and Dr. F. Yoshida of this Institute for their valuable suggestions. He also wishes to express sincere thanks to Dr. M. Mogi, President of this Institute, for his support and encouragement.

Studies on the Activities of Bacteria in Soy Sauce Brewing*

Part IV. Proteases, Transaminases of *Pediococcus soyae*, nov. sp., and its Numerical Transition in Soy Mash

By Kenji Sakaguchi

The extracellular proteinase and peptidase of *Pediococcus soyae*[1] is not found in its growing culture solution and culture filtrate. In the cell extract, weak activities of proteinase and peptidase are observed.

Weak transaminase and reductive amination activities of producing glutamic acid from α-ketoglutaric acid are estimated in the resting cell suspension and also in the crude cell extract. The potent amino group doners are; alanine, leucine, lysine, aspartic acid and ammonium chloride.

Starch fermentation by *Ped. soyae* strain *d* 6 is confirmed.

The numbers of lactic acid bacteria in soy koji are about 0.1–3 billion per gram and are much numerous than aerobic spore forming bacteria. The majority of them are salt sensitive *Streptococci* and *Lactobacilli*, but fewer salt tolerant *Pediococcus soyae* also exist. In the soy mash, salt sensitive lactic acid bacteria decrease rapidly within a period of two months. On the contrary, *Ped. soyae* grows well in the 18% salted mash, starting at 10^2–10^3/ml, reaching to 10^8–10^9/ml at the 4th month of brewing. Thereafter the viable counts of *Ped. soyae* gradually decreased and settled down to about 10^4/ml after 9 months of brewing. The pH value in soy mash was lowered while *Ped. soyae* was 10^6/ml or more.

Soy sauce brewing is very characteristic in microbiological industry because the principal biochemical reaction is the proteolysis of vegetable proteins, subsequently many reactions concerning peptides and amino acids. In the previous papers,[1,2] it was concluded that the most active bacteria in soy mash is a lactic acid bacterium i.e., *Pediococcus soyae* nov. sp.. It is generally admitted that the proteolytic enzymes of lactic acid bacteria is not recognized or very weak. Nevertheless, endocellular proteolytic enzymes have recently bean detected in several lactic acid bacteria. Berger[3] reported on the properties of intracellular peptidases of *Leuconostoc mesenteroides*. Nelson and his colleagues have further investigated the proteolytic enzymes of cheddar cheese originating in the extracellular and endocellular proteinases and peptidases of *Lactobacillus casei*[4] and *Streptococcus lactis*.[5] Other investigators have also conducted studies on these cheese proteinases.[6,7,8]

Concerning the transaminase activity of lactic acid bacteria, the existence of this activity has been reported on *Streptococcus faecalis*.[9,10,11] *Leuconostoc mesenteroides*[10] and *Lactobacillus ar-*

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1) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 353 (1958).
2) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*. **22**, 345 (1958).
3) J. Berger, et al., *J. Biol. Chem.*, **124**, 395 (1938), *J. Bact.*, **36**, 521 (1938).
4) E. Brandsaeter and F. E. Nelson, *J. Bact.*, **72**, 68, 73 (1956).
5) W. C. van der Zant and F. E. Nelson, *J. Dairy Sci.*, **36**, 1212 (1953), **37**, 795 (1954).
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9) J. T. Holden et al., *J. Biol. Chem.*, **191**, 559 (1951).
10) A. Meister, *Adv. in Bnzymol.*, **16**, 185 (1955).
11) A. Meister, *J. Biol. Ghem.*, **195**, 813 (1952).

abinosus.[10,11] However, the experimental method applied in these reports was the replacing of certain amino acids with corresponding keto-acids in a complete synthetic medium in which test organisms were inoculated and growth responses examined. In the case of *Ped. soyae*, a peptide-like nutritional factor (P-factor)[1,21] is required for growth, and the replacing method cannot be applied to this. In this paper, attempts were made for the detection of extracellular proteinases and also to detect the cooperation with *Aspergillus* proteinases. The endocellular proteinase and peptidase, the transaminase of this organism were also studied. Starch fermentation was confirmed by a weak starch fermenting strain *Ped. soyae d* 6.[1]

This paper also diels with the species and the viable cell numbers of lactic acid bacteria in soy koji, and the numerical changes of salt sensitive and salt tolerant lactic acid bacteria throughout a full-year of soy mash brewing.

EXPERIMENTAL

Measurement of proteinase activity. An application of Anson's[12] method reported in the preceding paper[2] was adopted. The substrate for the proteolysis was Hammersten cascin (Merck), which was enzymated at pH 6.5.

Extraction of Intracellular enzymes. Based on the reports of Brandsaeter and Nelson concerning endocellular proteases of *Lactobacillus casei*,[4] an eight-day culture of *Ped. soyae dl* 2 or *d* 4 was centrifuged, the cells were harvested and washed 3 times with a phosphate buffer of pH 7.0. Then the cells were destroyed by a 10 KC sonic oscillator (Kubota) for thirty minutes and the cell debris were centrifuged by rotation of 6000 per minute, for thirty minutes. The supernatant could be stored for several days at −30°C in vacuum flask cooled with dry ice.

Measurement of peptidase activity. Two millilitres of the supernatant of *Ped. soyae* cell extract was added to 5 ml of the substrate solutions buffered with M/15 phosphate pH 6.5. The substrates were 400 μM of glycyl-L-leucine (Nutritional Biochemicals Corp.), L-alanyl-glycyl-glycine (Delta Chemical Works). The mixed solutions were incubated for ten hours at 37°C, and the liberated amino acids were tilrated with a N/50 alcoholic solution of potassium hydroxide, following micro-alcoholic titration[4,13] by the method of Grassman and Heyde.

Measurement of transaminase activity.[14] In a total volume of 1.5 ml, 25 μM of α-keto-glutaric acid, 50 μM of each L-amino acid, 0.75 ml of pH 7.0 phosphate buffer, and 4.9 mg (as dry matter) of washed cell suspension or the corresponding volume of the maceration sap of cells were thoroughly mixed, and enzymated for four hours at 37°C. After lapse of enzymation period the flasks were placed into a boiling water bath for five minutes so as to arrest the reaction. The produced L-glutamic acid was measured manometrially using dried cells of *Escherichia coli* Najjar's strain[15].

Starch fermentation. *Ped. soyae d* 6[1] was subcultured for five days in the glucose supplemented nutrient broth, and then inoculated in the following broth: Soluble starch (Merck) 12 g, polypepton (Takeda) 3 g, beef extract (Difco) 1.2 g, dipotassium phosphate 2 g, salt 2 g, calcium carbonate 8 g and brom thymol blue. With occasional neutralization by concentrated sodium hydroxide solution, the inoculated broth was incubated anaerobically for four months at 30°C, and analyzed according to the routine method[1].

Viable counting of lactic acid bacteria in soy koji and in soy mash. One gram of the soy koji or 1 ml of the soy mash was suspended in 50 ml of sterilized glucose supplemented nutrient broth (For dilution of soy mash, salt was added to 17% in the broth.); 0.5 ml of the broth was pipetted and transferred aseptically to the next flask containing 50 ml of the broth. From the suitably diluted suspension, 0.5 ml was pipetted, inoculated into the isolation medium descibed in the previous paper, and cultured in test tube. In order to inhibit the surface growth of molds and aerobic bacteria, the uninoculated medium was poured into the tube after the inoculated agar became solid. The added antifungal antibiotic eurocidin inhibited the growth of molds and yeasts[20]. In order to count the viable numbers of *Ped. soyae*, the medium was salted to 15%. Viable numbers of salt-sensitive lactic acid bacteria were represented by the difference in colony counts between the 15% salted and the unsalted medium.

21) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*, in press (Part VII).
12) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).
13) M. Grassmann and W. Heyde, *Z. physiol. Chem.*, **183**, 32 (1929).
14) I. Shiio et al., *J. Biochem.*, **43**, 779 (1956).
15) V. A. Najjar, *J. Biol. Chem.*, **175**, 281 (1948).

RESULTS

Proteolysis by *Pediococcus soyae*.

Detection of proteolytic activity in the growing culture. Ten strains of *Ped. soyae* isolated from the soy mash, and, for comparison, *Lactobacillus arabinosus* and *Streptococcus faecalis* R were cultured in the following medium for twelve days at 30°C and the degradation of added milk casein was investigated. The medium composition was: Hammersten casein 1%, yeast extract (Difco) 0.5%, polypepton (Tadeda) 0.1%, glucose 2% and calcium carbonate 0.5%. The free amino group in the culture solution was estimated by Van Slyke's volumetric gasmetry. The nitrogenous compounds which were soluble in 0.4 M trichloroacetic acid were also determined by Kjeldahl's method.

Although the cheddar cheese lactic acid bacteria, *Lactobacillus casei*,[4] was reported to hydrolyse milk casein in growing culture, *Ped. soyae* did not suggest any proteolytic activity in the medium, and the added pepton was found to be only consumed. (Table I)

Table I. Decomposition of Milk Casein by Growing Culture of *Ped. soyae*

Strain	Free Amino N mg/ml (Van Slyke)	TCA soluble N mg/ml (Kjeldahl)
Control	0.47	1.40
d 1	0.38	1.30
d 2	0.30	1.20
d 3	0.37	1.25
d 4	0.35	1.30
d 5	0.44	1.25
d 6	0.34	1.35
d 7	0.37	1.35
d 9	0.25	1.35
dl 1	0.37	1.30
dl 2	0.39	1.35
L. arabinosus	0.35	1.20
Str. faecalis	0.36	1.35

Detection of proteolytic activity in the culture filtrate. The supernatant (7000 r.p.m., for fifteen minutes) of the culture solution was used as the enzyme solution and proteolytic activity on the following substrates was examined: Hammersten casein, its partial hydrolysates by *Asp. sojae* proteinase, *Bacillus subtilis* crystalline proteinase (Nagase Ind.) and hydrochloric acid. Enzymation was continued for eighteen hours at 30°C with the addition of thymol and penicillin. There was no increase of phenolic amino acids on any substrates beyond the range of measurement error (Table II). Therefore, extracellular proteolotic enzymes of *Ped. soyae*, both proteinase and peptidase, were not detected at pH 6.0 and 4.0.

Table II. Detection of Extracellular Proteinase

Substrate	Increase of Optical Density	
	pH 4.0	pH 6.0
Casein	−0.005	−0.003
Partial Hydrolysate of Casein by *Asp. sojae* Protease	0.001	0.005
Partial Hydrolysate of Casein by Crystalline *Bac. subtilis* Protease	0.004	0.002
Partial Hydrolysate of Casein by 2 N HCl	0.005	0.003

The assumption that lactic acid bacteria, though impotent in proteolytic activities by themselves, may strengthen or support the proteolytic activity of *Asp. sojae* was also investigated. The culture filtrate of *Ped. soyae* was added to an equivalent volume of *Asp. sojae* mold bran extract (1 g per 20 ml) which was enzymated with Hammersten casein at pH 4.0 for four hours at 30°C. The results shown in Table III indicate that there is no significant difference discernible whether the culture filtrate of *Ped. soyae* is added or not.

Table III. Detection of the Proteolytic Cooperatin with *Asp. sojae* Proteinase

Enzyme Solution	Increase of Optical Density	
	Example 1	Example 2
Ped. soyae	−0.009	−0.007
Crude Extract of *Asp. sojae* Mold Bran	0.047	0.165
Crude Extract of *Asp. sojae* Mold Bran + *Ped. soyae*	0.046	0.160

Endocellular proteinase and peptidases. Brandsaeter and Nelson reported on the protelytic strain of *Lact. casei*,[4] and also on the existence of a rather weaker endocellular proteinase (opt. pH 5.0–7.0) and a stronger endocellular peptidases (opt. pH 6.0–8.0). Similar results of *streptococcus lactis*[5] were obtained by Van der Zant and Nelson. *Ped. soyae* is also a distinguished lactic acid bacterium in regard of playing a role in proteolytic fermentation, hence endocellular proteolytic enzymes are to be studied.

The cell free extract was filtered with Seitz's filter and 1 ml of the sterile filtrate was added to 2 ml of autoclaved Hammersten casein solution at 30°C, pH 6.5. The results shown in Table IV clearly indicate the existence of endo-

TABLE IV. INTRACELLULAR PROTEINASE OF *Pod. soyae*

Incubation Days		Transmittance %	Optical Density	Increase of O.D.
1	Enzyme Solution	88.2	0.054	0.039
	Casein+Enzyme Solution	80.2	0.094	
7	Enzyme Solution	81.3	0.088	0.125
	Casein+Enzyme Solution	61.1	0.213	

cellular proteinase activity, but by reflecting on the concentration of the enzyme solution and the length of enzymation time the weakness of the activity is affirmable.

The endocellular peptidases were estimated in the same manner as in the enzymation for ten hours at 37°C, pH 6.5. As shown in Fig. 1, about 20–25% of the substrates were splitted after ten hours enzymation. Activity was stronger than endocellular proteinase activity, but resulted to be weaker than the endocellular peptidases of *Lact. casei* in cheddar cheese.[4]

Transaminase and glutamic acid dehydrogenase. Transaminase activity has been observed in many microorganisms.[10,16] Among lactic acid bacteria, its existence in *Streptococcus faecalis*,[9,10,11] *Leuconostoc mesenteroides*,[10] *Lactobacillus arabinosus*[10,11] have been reported. In spite of this, the experimental method of most of these reports is as started in the preface the replacing method which can't be applied to *Ped soyae*. In this experiment, intact cell suspension and crude cell extract by sonic oscillation were employed as the enzyme solution.

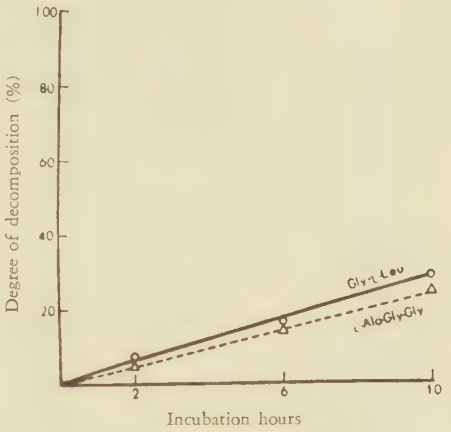

FIG. 1. Intracellular Peptidase of *Ped. soyae*

The relative values of the produced L-glutamic acid against added α-keto-glutaric acid are listed in Table V. Though the activities were in general low, rather broad specificities were observed in many amino acids. The potent amino group doners were: alanine, leucine, valine, histidine, lysine, tyrosine, aspartic acid, cystine and ammonium chloride. It is noteworthy that ammonium chloride was the most potent amino group doner, thus, the activity of glutamic acid dehydrogenase was presumed.

Soy mash is an almost saturated solution composed of amino acids, and the existence of transaminase activity in the most active bacteria, *Ped. soyae* provides an important suggestion for the glutamic acid production in soy mash. That is, another synthetic way for producing glutamic acid from sugar-materials is to be considered besides the hydrolytic way of protein-material. Nevertheless, the real action of *Ped. soyae* transaminase in soy mash is doubtful because of its low pH value and high salt

16) H. Katagiri, et al, read at the General Meeting of Agr. Chem. Soc. Japan, on 4th May, 1958.

TABLE V. TRANSAMINASE ACTIVITY OF *Ped. soyae*

Doner of amino group	Relative value of L-glutamic acid formed	
	Intact cell %	Crude extract %
glycine	0.1	1.3
alanine	0.3	4.0
isoleucine	1.1	2.0
leucine	1.0	2.9
valine	1.2	2.3
arginine	1.2	1.7
histidine	1.7	2.0
lysine	1.3	3.0
serine	1.0	2.3
threonine	1.2	2.3
phenyl alanine	1.1	0.0
tyrosine	1.5	2.4
trytophane	0.6	−0.5
aspartic acid	1.0	3.9
proline	0.1	1.9
cystine	0.0	2.6
cysteine	−0.3	0.2
methionine	−0.1	−0.2
NH_4Cl	1.4	4.3

content. In the author's unpublished data,[17] about 30% of α-keto-glutaric acid added to the one-year old brewed soy-mash was converted into L-glutamic acid with the addition of ammonium phosphate. This phenomenon was however, not observed in the fresh soy mash in which *Ped. soyae* was actively growing. Therefore, it is probable that either yeasts or any other unidentified microorganisms might play a role in the transamination of soy mash.

Starch fermentation by *Ped. soyae* strain *d* 6. In the previous paper, *Ped. soyae* strain *d* 6 and *dl* 1 revealed weak starch fermentablity after one month's anaerobic incubation at 30°C. The starch fermentation by lactic acid bacteria was rarely observed except that by the well-known starch fermenters *Streptococcus bovis*[18] and *Str. equinus*.[19]

Fermentation began after one month's lag period and thereafter smoothly proceeded until almost all of the added starch was consumed, which was after four month's fermentation. From 10.9 g of consumed starch, 1.44 g of ethanol, 3.55 g of acetic acid and 4.28 g of dextrorotatory lactic acid were produced. Fermentation tests were also conducted on 7% and 15% salted broth, but no growth was observed in both cases. Amylase tests for this strain were negative on both α-amylase and β-amylase activity.

Lactic acid bacteria in soy koji. In order to investigate the source and the infecting route of *Ped. soyae* into soy mash, lactic acid bacteria in soy koji were isolated and viable numbers per gram were counted.

The addition of an antifungal antibiotic, eurocidin,[20] could effectively inhibit the growth of the koji mold (*Asp. sojae*) and contaminating yeasts. The tube culturing in which a low rH value was retained by the addition of sodium thioglycollate, only permitted the growth of lactic acid bacteria amongst other microorganisms. The tube culture with the aid of 0.1% sodium thioglycollate provided a more perfect anaerobic condition than plate culturing in a vacuum jar, and resulted in much more colony counts of lactic acid bacteria.

Viable numbers of lactic acid bacteria in soy koji monufactured in the factory of Noda Shoyu Co. were from 0.1 to 3 billion per gram, and about ten-fold more than the viable counts of the *Bacillus subtilis* group, which were formerly accepted as the main contaminants in soy koji or rice koji. From the colonies grown in the tubed agar, 46 strains were isolated and inspected microscopically.

Gas producing lactic acid bacteria were few, and short-chained short rods ($0.3\,\mu \times 1.0\,\mu$, supposed to be a *Lactobacillus* sp.,) and the short-chained cocci (0.5–0.7 μ in diameter, supposed to be a *Streptococcus* sp..) prevailed. These two types of lactic acid bacteria could grow only in 7% salted medium, but not in 15% salted medium. The two salt-sensitive strains isolated from fresh soy mashes[1] had identical forms

17) K. Sakaguchi, unpublished.
18) Shermen, *Bact. Rev.*, **1**, 57 (1957).
19) Andrewes and Horder, *Lancet*, **2**, 712 (1906).
20) K. Sakagushi, *J. Agr. Chem. Soc. Japan*, **28**, 758 (1954); *Rep. Noda Inst. Sci. Res.*, **2**, 25, (1958).

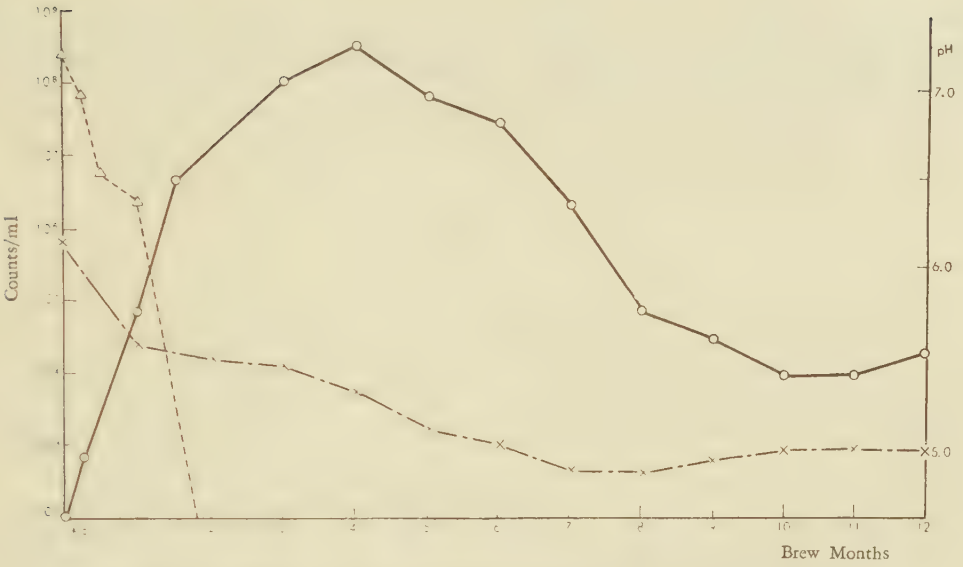

FIG. 2. Change of Viable Counts of Lactic Acid Bacteria in Soy Mash
—○— *Pediococcus soyas* —△— Salt sensitive lactic acid bact. —×— pH

with those in soy koji.

Four strains of tetra formed *Pediococci* were found from the isolated strains, and grew well in 15% salted medium. It is therefore concluded from these experiments that the origin of *Ped. soyae* in soy mash is primarilly due to soy koji, which is probably originated to the covering microbial atmosphere for a period of three hundred year's soy sauce brewing in Noda City.

Numerical transition of *Ped. soyae* and other lactic acid bacteria in soy mash. On the soy mash collected from the Seventh Factory of Noda Shoyu Co. throughout one years brewing, changes of viable counts in *Ped. soyae* and salt sensitive lactic acid bacteria were examined. The number of *Ped. soyae* was represented by the colony counts grown in 15% salted agar. The number of salt sensitive lactic acid bacteria was represented by the difference of colony counts between salted the unsalted media.

Fig. 2 gives the result. Salt-sensitive lactic acid bacteria grown in the soy koji diminished in their viable numbers, but about 10^6–10^7 cells per millilitre survived within one month's brewing in a 18% salted circumstance. These did not multiply, but their physiological activities may still have been active depressing the pH of the soy mash. After one and a half month's brewing at about 30°C, *Ped. soyae* exceeded in numbers, while salt-sensitive bacteria diminished and the pH of the mash was lowered due to the rapid growth of the organism. On the fourth month of brewing, viable numbers of *Ped. soyae* reached to its maximum, namely 260 million per millilitre. Thereafter colony counts slowly decreased, and settled down to about 10^4 counts per millilitre. The pH of the mash was minimum on the seventh or eighth month reaching to pH 4.85 (measured with a glass electrode pH meter). When the count of viable numbers of *Ped. soyae* was 10^5/ml or less, the pH of the soy mash gave rise and came up to pH 5.0 at the end of brewing.

Acknowledgements. The author wishes to express his hearty thanks to prof. T. Asai and Prof. K. Kitahara for their kind guidances. Thanks are also due to Dr. K. Sakaguchi, Prof.

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A part of this report was read at the General meeting of the Soc. Agr. Chem. Japan on 3rd, May, 1958.

Studies on the Activities of Bacteria in Soy Sauce Brewing*

Part V. The Effects of *Aspergillus sojae, Pediococcus soyae, Bacillus subtilis* and *Saccharomyces rouxii* in Purely Cultured Soy Sauce Brewing

By Kenji Sakaguchi

The soy koji mold *Aspergills sojae* is purely cultured on the materials of soy koji, and soaked in the sterile concentrated sodium chloride solution (26%). To it, *Ped. soyae* and *Sacch. rouxii* are aseptically inoculated and their roles playing in soy sauce brewing are examined. *Bacillus subtilis* is added in the form of wheat bran culture into the purely cultured soy mash and its influencies are observed.

The appropriate addition of the bran culture of *Bac. subtilis* favors for the development of necessary aroma, and eliminates the polypeptide-like turbidity in soy sauce. The growth of *Ped. soyae* in soy mash stimulates the formation of organic acids, and makes the mash acidic which is necessary for the sound fermentation, removes undesirable flavors, and adds the indispensable good flavors for soy sauce. But the rapid lowering of pH results in the depression of *Aspergillus* Alkaline Proteinase activities[1,2,3], followed by its inactivation, thus the percentage of solubilization of the protein material decreases. The colour of soy sauce also becomes lighter. *Sacch. rouxii* produces ethanol, and cooperating with *Ped. sojae*, plays an important part for developing the soy sauce flavor. *Asp. sojae* is the most important microorganism for soy sauce brewing. Almost all the essential enzymes for soy sauce brewing originates in *Asp. soyae*, as a result, fundamental chemical components, aroma and flavor are developed by enzymes from this mold. The soy sauce brewed by *Asp. sojae* alone, however, is not equal to the one which is made with mixed organisms, but it is only the mother body of soy sauce. The solubilization ratio of the protein material has also reached to the maximum without the aid of other microorganisms. However, the production of organic acids and ethanol is insufficient, polypeptide-like turbidity in soy sauce has remained, and aroma and flavor are not as well as the normally brewed soy sauce.

By addition of bacterial and yeast cultures, attempts for improving the quality of soy sauce were frequently reported. But they were all dealing with open natural cultures, and no proper roles of individual microorganisms were extensively studied. In this experiment, the important four microorganisms relating to the soy sauce brewing were all purely cultured and added, and their roles were elucidated by chemical analysis, by enzyme activities and by sensuous testing. The four important microorganisms dealt with were; *Aspergillus sojae*[4,5], *Pediococcus soyae*[6], *Bacillus subtilis*[1,7,8] and *Saccharomyces rouxii*[9]. The other isolated micro-

* Reprinted by permission of Agr. Chem. Soc. Japan from Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 2.

1) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 345 (1958).

2) T. Mizunuma and N. Iguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 35 (1957).

3) W. G. Crewther and F. G. Lennox, *Aust. J. Biol. Sci.* **6**, 428 (1953).

4) K. Sakaguchi and K. Yamada, *J. Agr. Chem. Soc. Japan*, **20**, 64, 141 (1944).

5) K. Yamamoto, *Bull. Agr. Chem. Soc. Japan*, **21**, 308, 313, 319 (1957).

6) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 353 (1958); **23**, 22 (1959).

7) Y. Ishimaru, *J. Agr. Chem. Soc. Japan*, **9**, 859, 953, 1143 (1933).

8) K. Matsumoto, *J. Soc. Brewing Japan*, **21** (1926), **22** (1927), **23** (1928), **24** (1929).

9) H. Onishi, *Bull. Agr. Chem. Soc. Japan*, **21**, 137, 143, 151 (1957).

organisms relating to soy suace brewing; namely lactic acid bacteria growing in soy koji[6], *Brettanomyces*[7,8] and *Torulopsis*[9,10,11] appearing in the late stage of soy mash fermentation, were excluded in this study because of their meager influences on the brewing.

EXPERIMENTAL

Preparation of purely cultured soy mash. A large two necked Komagome type flask (Fig. 1) was used for the pure culture of soy koji. To it, 49.5 g of defatted granular soy bean, 24.2 g of parched and cracked wheat grain and 7.9 g of wheat bran were added and autoclaved for forty minutes at 1.5 atm/cm^2. The defatted soy bean and wheat bran were pre-treated by spraying with boiling water. The sterilized materials were inoculated with *Aspergills sojae* spores and incubated for three days at 30°C. After the whole materials were covered with mycelia and spores, the soy koji was aseptically transfered to the bottle illustrated in Fig. 2, and soaked with 180 ml of autoclaved brine containing 26.00% sodium chloride. The concentration of salt was too high in this case, and it should be corrected to about 24%. The salt used was the Arabian bay salt, and its sodium chloride content was about 91%. After the soaking, the bacterial contamination was inspected using eurocidin media[12]. The bacteria-free bottles were incubated at 30°C for fwo and half months. Time to time the air was bubbled through the longer cotten tube into the bottle to stimulate the growth of yeast which was inoculated latter.

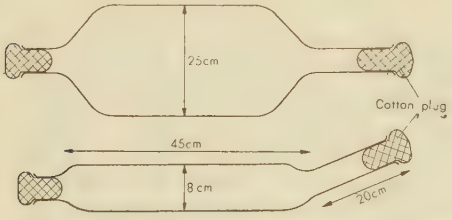

FIG. 1. The Two Necked Komagome Type Flask for the Cultivation of Purely Cultured Soy Koji.

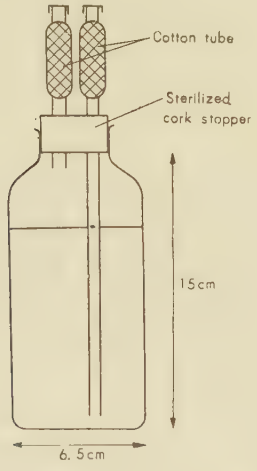

FIG. 2. The Bottle for the Brewing of Purely Cultured Soy Mash.

Inoculation of *Pediococcus soyae*. *Ped. soyae dl* 2[6] was subcultured in 5 ml of the broth, containing yeast extract (Difco) 0.3%, polypepton (Takeda) 0.5%, glucose 1.0%, dipotassium phosphate 0.5% and sodium chloride 7.0%. This culture was incubated anaerobically from three to six days at 30°C, then poured into the purely cultured soy mash three days after the soaking of soy koji.

Inoculation of *Saccharomyces rouxii*. *Sacch. rouxii* strain No. 7[13,9] isolated from the soy mash was subcultured in 5 ml of rice koji water (Bllg 10°) containing 7% sodium chloride for three or six days at 30°C. This culture was inoculated into the purely cultured soy mash on the same day as *Ped. soyae*.

Addition of bran cultured *Bacillus subtilis*. Five grams of wheat bran, 1 g of soy bean powder and 5 ml of water in 150 ml Fernbach flask was autoclaved at 1.5 atm/cm^2 for forty minutes. Srong proteinase producers, *Bac. subtilis var. Biotecus* A–538 (Nagase industries Co.), *Bac. subtilis* strain Fn and H–127–H (Institute of Applied Microbiology, University of Tokyo) were inoculated and cultured respectively for three days at 30°C. The resulted each bacterial bran was added into the purely cultured soy mash at the time of soaking with brine.

Mixed culture of *Asp. sojae* and *Bac. subtilis var. Biotecus* A-538. *Asp. sojae* was inoculated on the autoclaved soy koji materials, and after one day's culture, *Bac. subtilis var. Biotecus* A–538 was inoculated. The incubation was continued for two days thereafter.

Brewing of the factory soy koji. The soy koji was supplied from the Seventh Factory, Noda Shoyu Co..

10) H. Onishi, read on the General Meeting of the Soc. Agr. Chem. Japan on April, 1957.
11) M. Mogi and H. Onishi, *J. Ferment. Technol.*, **33**, 396 (1955).
12) K. Sakaguchi, *J. Agr. Chem. Soc. Japan*, **28**, 758 (1954), *Rep. Noda Inst. Sci. Res.*, **2**, 25 (1958).
13) H. Onishi, unpublished.

Four hundred grams of the soy koji was soaked with 700 ml of crude concentrated brine containing 22.60% sodium chloride. The mash was incubated for three months at 30°C in the same manner as the purely cultured soy mash.

Measurement of hydrogen ion concentration. The pH Test Papers (Toyo Roshi Co.), Brom Cresol Purple and Brom Cresol Green were calibrated with a Beckman pH meter and were used.

Titration of total acid. One millilitre of soy sauce was diluted to 50 ml with distilled water, and titrated with N/20 sodium hydroxide to pH 7.0 using a Beckman pH meter Model G. The value was expressed by milligram of lactic acid per ml of soy sauce[14].

Measurement of proteinase activity. The method described in the previous paper[1] was followed.

Assay of free amino nitrogen[14,15] Formol nitrogen was titrated potentiometrically according to the method of Sakasai and Yokotsuka.

Assay of ammonium. The soy sauce was diluted 50 fold, and Conway's method of microdiffusion analysis[16] was applied.

Assay of polypeptide[17]. To 10 ml of soy sauce, 25 ml of dist. water, 5 ml of 10% sodium tungstate solution and 10 ml of 1 N sulfuric acid were added successively and polypeptides in soy sauce were precipitated in a refrigerator over night. The precipitate was collected by centrifugation, washed with 10% tungsic acid, and the nitrogen content of the material was measured by Kjeldahl's method.

Assay of glutamic acid. The soy sauce was diluted 1000 fold, and glutamic acid content was bioassayed using *Lact. arabinosus* as test organism[18,19].

Measurement of α-amylase activity. The unpasteurized soy sauce was diluted ten fold with M/15 phosphate buffer pH 6.0, and used as an enzyme solution. Two millilitre of the enzyme solution was poured into 10 ml of 0.4% soluble starch solution (pH 6.0) and incubated at 30°C. A half milliliter of the mixed solution was taken out at five minutes intervals, and poured into 2 ml of the 2 N I_2 solution. The time when the blue colour of starch-I_2 reaction has disappeared, was recorded.

Assay of reducing sugars. Fehling-Lehmann-School's method was followed.

Colour intensity. The soy sauce was diluted 12 fold, and measured by an electrophotomer (Erma) with the transmission light through 420 mμ filter. The value was given by the optical density.

RESULTS AND DISCUSSION

As shown in the Table, 10 lots and 18 bottles of purely cultured soy sauces were prepared and brewed for two and half months at 30°C, and their enzymic activities, chemical compositions, solubilization ratios of materials, flavors and aromas were examined. Since all microorganisms added were purely cultured, the roles of each of them were clearly shown in this table. The tested organisms were; *Asp. sojae strain KS.*[4,5], three strains of strong protease producers *Bacillus subtilis var. Biotecus* A–538 (Nagase Industries Co.), *Bac. subtilis* strain Fn and H–127–H (Institute of Applied Microbiology, University of Tokyo), *Pediococcus soyae*[6] strain *dl* 2 and *Saccharomyces rouxii* No. 7[9,10,11].

Asp. sojae is the so called soy koji mold, and it is the most important microorganism for soy sauce making. The organism produces proteases, amylases, other important enzymes for soy sauce brewing, and also aromas and flavors. *Bacillus subtilis* grows only in the koji making stage. *Pediococcus soyae* is the only active bacteria in soy mash, and produces lactic acid. *Saccharomyces rouxii* also grows well in soy mash and ferments only glucose but no other sugars.

The combinations of microorganisms were; *Asp. sojae* koji alone, *Asp. sojae* koji plus *Bac. subtilis* brans, mix-cultured koji of *Asp. sojae* and *Bac. subtilis*, *Asp. sojae* mash plus *Ped. soyae* culture, *Asp. sojae* mash plus *Sacch. rouxii* culture, *Asp. sojae* mash plus *Sacch. rouxii* and *Ped. soyae* culture, *Asp. sojae* koji plus *Bac. sub. var. Biotecus* bran plus *Sacch. rouxii* culture factory soy koji.

The tasting of the brewed soy sauce were entrusted to four members of tasting committee in Noda Shoyu Co.. The hydrogen ion con-

14) T. Sakasai and T. Yokotsuka, *J. Agr. Chem. Soc. Japan*, **32**, 144 (1958).
15) M. S. Dunn and A. Loshakoff, *J. Biol. Chem.*, **113**, 395, 691 (1936).
16) E. J. Conway, Microdiffusion Analysis and Volumetric Error. p. 75 (1950).
17) S. Hori et al., *J. Agr. Chem. Soc. Japan*, **30**, 515 (1952).
18) T. Tsunoda, Z. Ishizuka et al., *ibid.* **26**, 478 (1952).
19) E. E. Snell, *Nutrition Abstr. and Rev.*, **16**, 497 (1946-47).

ANALITICAL AND TASTING DATA OF

Related microorganisms	NaCl concentration %	pH after 7 days	pH after 13 days	pH after 2.5 months	Total acid as lactic acid mg/ml	Proteinase activity after 7 days 2.5 Months pH 3.5	pH 7.0	pH 10.0	pH 7.0	Total nitrogen in soy sauce 10 mg/ml, %	Utilizing ratio of total nitrogen %	Amino N / Total N %
	20.34	6.6	7.0	5.5	7.4	0.1	4.5	5.1	4.0	1.53	77.7	56.9
Asp. sojae.	20.27	6.4	6.6	5.3	7.9	0.3	4.1	5.2	3.5	1.50	76.3	56.0
	20.43	6.6	6.4	5.3	7.2	0.1	3.5	3.9	3.1	1.47	74.5	55.6
Asp. sojae, Bac. sub. var. Biotecus.	20.36	6.6	6.4	5.3	6.4	0.0	5.2	3.8	5.0	1.54	77.8	56.6
	20.50	6.4	6.4	5.5	5.0	0.1	3.9	4.7	4.3	1.47	73.7	58.6
Asp. sojae, Bac. sub. Fn	20.30	6.6	7.0	5.7	4.1	0.1	4.8	3.9	2.8	1.50	76.0	57.5
	20.36	6.8	6.8	5.3	5.4	0.1	4.8	3.1	3.2	1.51	76.6	55.6
Asp. sojae, Bac. sub. H–127–H	20.59	6.8	6.8	5.5	4.5	0.0	2.6	2.6	2.3	1.46	73.1	57.3
	20.41	6.6	6.8	5.7	3.9	0.0	3.1	2.9	3.4	1.45	73.3	56.2
Asp. sojae, Bac. sub. var. Biotecus. (Mixed culture)	20.54	6.9	7.2	5.3	6.3	0.2	4.8	4.0	3.3	1.50	75.6	57.6
	20.46	6.9	7.2	5.3	7.2	0.6	5.6	4.2	4.2	1.54	77.5	54.7
Asp. sojae, Sacch. rouxii No. 7.	20.62	6.8	6.8	5.5	4.7	0.0	3.3	3.7	3.0	1.41	70.3	58.2
	20.52	6.8	7.0	5.5	5.0	0.1	3.4	3.5	3.0	1.46	73.5	57.5
Asp. sojae, Ped. soyae dl 2	20.10	6.4	4.9	4.7	11.7	0.0	3.6	5.1	0.6	1.30	66.5	58.4
	20.35	6.6	4.9	4.5	11.2	0.1	3.0	3.8	0.5	1.18	59.8	61.0
Asp. sojae, Ped. soyae dl 2 *Sacch. rouxii* No. 7.	20.50	6.4	5.6	4.7	10.5	0.2	2.9	3.4	0.5	1.30	65.3	54.9
Asp. sojae, Bac. sub. var. Biotecus, Ped. soyae, Sacch. rouxii	20.33	6.6	4.6	4.7	10.9	0.0	6.1	4.6	1.3	1.40	71.0	58.7
Factory soy koji.	17.78	6.0	5.6	4.5	12.2	2.0	6.3	5.4	1.7	1.37	——	58.7

centration of the soy mash was measured on seventh, thirteenth day, and two and half months after the soaking of soy koji. The proteinase activity of the soy mash juice was measured at three pH values, 3.5, 7.0 and 10.0 on the 7 th day after the soaking, and two and half months later, at pH 7.0.

On the samples brewed for two and half months, the other properties of the products were examined, because, the fermentation and maturing were usually concluded in three months when the soy sauce brewing was conducted in the warmed chamber at 30°C, and the product was almost identical with the normal soy sauce brewed for a year under uncontrolled temperature.

Roles played by *Asp. sojae*. The soy koji mold *Asp. sojae* was undoubtedly the most important organism for soy sauce brewing. By its biochemical actions during koji making stage, and by its enzymatic actions in soy mash, the mother body of the soy sauce was produced. As shown on the table, the percentage of solubilized proteins (mainly soy bean protein) into the soy sauce (The value is called as utilizing ratio of total nitrogen.) reached to the maximum without the aid of other microorganisms. The proteinase activity of fresh soy mash juice also did not fall below other mixed cultured soy mashes. This provides an evidence for the

PURELY CULTURED SOY SAUCES

$\frac{NH_3\text{-}N}{Total\ N}$ %	Turbidity in Soy Sauce	$\frac{Polypeptide\ N^{17)}}{Total\ N}$ %	$\frac{Glutamic\ acid\text{-}N}{Total\ N}$ %	α-amylase activity (Starch-I_2) min.	Reducing sugars %	Alcohol %	Utilizing ratio of starch %	Color (429 mμ Optical Density	Tasting
19.0	+	13.4	8.50	35	3.41	0.28	27.2	0.98	Mother body of soy sauce flavor, but not the soy sauce flavor itself. Lacked in acid taste.
16.4	+	10.5	8.33	38	3.01	0.12	22.3	0.92	
18.3	++	11.3	8.16	35	2.97	0.13	17.7	0.89	Special drug-like odour as phenol or related substances.
23.5	−	11.3	8.44	25	2.58	0.10	18.3	1.03	
24.3	−	11.4	8.84	25	3.61	0.13	26.3	0.96	Drug-like odour was diminished.
19.7	−	12.4	8.60	33	3.14	0.17	23.9	0.88	Moderate flavor.
19.5	−	10.1	9.59	34	2.68	0.15	20.4	0.95	A necessary flavor for the soy sauce was developed.
19.0	−	11.0	8.84	34	2.99	0.21	23.1	0.93	Lacked in acid taste.
19.2	−	11.0	8.69	34	3.08	0.23	24.1	0.83	
19.5	−	11.6	8.48	<10	2.67	0.07	19.0	1.01	Bacillus odour.
18.3	±	11.5	8.18	<10	2.61	0.07	18.8	1.02	
19.4	±	11.4	8.58	35	1.97	0.61	21.5	0.82	Too strong flavor of yeast fermentation, flut this flavor might be necessary for the soy sauce.
18.7	±	12.0	8.36	40	1.86	0.64	21.3	0.82	
23.4	+	11.5	8.00	90	2.28	0.15	17.9	0.66	Odd smells were almost removed.
25.3	++	11.6	8.14	110	2.44	0.07	17.7	0.70	The essential flavor for the soy sauce was developed.
22.2	++	13.3	7.69	120	1.78	0.28	15.9	0.66	Smells almost identical with soy sauce.
23.7	++	10.9	7.71	70	1.67	0.25	14.9	0.72	Bacillus odour prevailed.
11.2	−	5.2	9.56	45	5.18	0.02	——	0.73	Yeast fermentation was insufficient.

previous report[1] that the proteinases in soy mash are almost *Aspergillus* origin.

Because of the lack of lactic acid bacteria, the pH of the mash was not rapidly lowered, and the proteinase activity was not so decreased even after two and half months incubation at 30°C.

The liberation of glutamic acid, α-amylase activity and solubilization ratio of starch in raw material were also normal.

It is apparent, however, that *Asp. sojae* is able to make *only the mother body* of soy sauce, and it is not the soy sauce in itself. The absence of lactic acid bacteria caused the acid insufficiency, and the pH was not lowered below pH 5.5 even after two and half months incubation at 30°C. If the mash is open cultured, it will be easily rotten by salt tolerant *Bacilli*[7,8], which is suppressed by the low pH value in the normal soy mash[6].

Although it was not a bad smell, soy sauce which was made with *Asp. sojae* alone had a different aroma and flavor from the normal one. It has a specific drug like odour, which was removed by the growth of *Ped. soyae* and *Sacch. rouxii*.

The absence of yeast resulted in the deficiency of ethanol, normal content of which was from 2.0% to 3.0% in the case of unpasteurized soy sauce.

A distinct fault of the purely cultured soy sauce which was made with *Asp. sojae* alone was the appearance of a large amount of polypeptide-like white precipitate in soy sauce. The precipitate appeared in soy sauce when it was refrigerated, heated, or even diluted by water. It is a troublesome character for an article of commerce.

Roles played by *Bacillus subtilis*. *Bac. subtilis* and other aerobic spore forming bacteria grow only in soy koji making stage, and their spores are surviving in soy mash[10]. These bacteria were formerly considered as a harmful contaminant in soy koji, but this experiment proved that the appropriate addition such *Bacilli* which does not produce unfavorable flavor, was useful and even necessary for soy sauce brewing. A specific drug-like odour of soy sauce brewed with *Asp. sojae* was diminished by the addition of bran culture of *Bac. subtilis*, further, the polypeptide-like turbidity of soy sauce also disappeared.

This improvement of soy sauce flavour is no wonder because there exists usually 10^6–10^8 cells of *Bacilli* per gram of the open cultured normal soy koji. But both mixed culturing of *Bac. subtilis var. Biotecus* A–538 with soy koji and the addition of its bacterial bran, gave soy sauce the so-called *Bacillus* odour. Therefore it is necessary to select strains and to regulate the excessive growth of *Bacilli*.

By addition of *Bacillus subtilis*, no increase of proteinase activity was observed, and the solubilization ratio of protein in raw material (represented by the utilizing ratio of total nitrogen) also did not increase. But the appearance of protein-like turbidity in soy sauce was effectively inhibited by addition of *Bacillus subtilis*. This phenomenon probably is due to the breaking of certain peptide bonds which is resistant to the attack of *Aspergillus* proteinases, so even the small quantity of different *Bacillus* proteinase plays an important role.

The α-amylase activity was increased by the addition of this bacteria. The utilizing ratio of starch was, however, not improved.

Roles played by *Pediococcus soyae*. In this experimental brewing, the salt concentration of purely cultured soy mash was too high by accident, 2 or 3% higher than the normal concentrations. Even in these high salt concentrations at 20.2–20.6%, *Ped. soyae* grew well and the mash was rapidly acidified to pH 4.9 within ten days after the inoculation. The hydrogen ion concentration of the other lots were pH 7.0–6.4 on the same day, and no acid production was observed by other organisms. Their final hydrogen ion concentration were pH 5.3–5.5, and have not reached to the normal pH value of 4.8–4.5 without the aid of *Ped. soyae*. The total acidity was also paralleled with the hydrogen ion concentration.

One of the most important characteristics of this lactic acid bacterium is the ability to form essential aromas and flavors for soy sauce. Its growth clearly eliminated the drug-like smell which was pronounced in the purely cultured soy sauce of *Asp. sojae*, and aromas became mild and well-matured. This activity of improving soy sauce aroma and flavor was so distinguished that the necessity of *Ped. soyae* for soy sauce brewing was indisputable despite some disadvantages of this organism described later.

It was mentioned that the growth of this microorganism lowered pH of the mash below pH 5.0, and protect the soy mash from spoilage by salt tolerant *Bacilli*. Another important effect of low pH value is to stimulate the growth of salt tolerant yeasts. Onishi[9] reported that, in 18% salted media, *Sacch. rouxii* could grow only in the range of pH 5.0–4.0. (The value was measured by the pH Test Pater (Tokyo Roshi Co.). Brom Cresol Green. When it was measured by a Beckman pH meter Model G, about 0.6 higher pH values were obtained in the case of highly salted solutions.) Without the growth of *Ped. soyae*, the growth and fermentation of yeasts is imperfect.

As stated above, *Ped. soyae* is indispensable for the soy sauce brewing, but its too early growth in soy mash decreases the yield of soluble

nitrogen in soy sauce. This microorganism has only the weak proteinase and peptidase in the cell, but had neither extracellular proteolytic enzymes nor the co-operating ability with *Aspergillus* proteinases[6]. On the other hand, the rapid growth of the lactic acid bacteria acidifies soy mash, and the optimal hydrogen ion concentration of *Aspergillus* alkaline proteinase[1,2,3,5,20] (pH 6.5–11.0) was soon missed. The proteinase activity of soy mash at pH 5.0 is about one tenth of that at pH 7.0. Moreover, the *Aspergillus* alkaline proteinase is rapidly inactivated because of the low pH of mash. The table shows that about eight tenth of the proteinase activity at pH 7.0 remained after two and half months brewing at 30°C in *Ped. soyae* omitted lots, while the lots with *Ped. soyae* kept only about two tenth of the activity. Accordingly, about 10% decrease of the solubilization ratio of total nitrogen in raw material was recognized on the lots.

The inactivation of *Asp. sojae* proteinases also caused the marked increase of the polypeptide-like precipitation in soy sauce, which was probably due to the imperfect digestion of protein.

The other actions played by *Ped. soyae* were; the increase of liberated ammonium, the inactivation of α-amylase of *Asp. sojae*, the decrease of the utilizing ratio of starch in raw material.

The colour intensity of soy sauce was markedly decreased by this organism, reaching to the light colour of the unpasteurized normal soy sauce.

In general, *Ped. soyae* is quite indispensable for forming the soy sauce aroma and flavour, and for conducting the sound brewing process in the open culture, but its acid production caused the inactivation of various enzymes of *Asp. sojae*. In order to overcome these unfavorable points, it is desirable to suppress the growth of *Ped. soyae* in the early stage of brewing, and after the enzymes of *Asp. sojae* have played larger parts of their roles, namely two or three months later, *Ped. soyae* should grow vigorously to acidify the soy mash and to develop the good flavor to soy sauce. From this view, it is reasonable that when soy koji is prepared and soaked in the brine during winter time, the resulted soy mash is superior both in its solubilization ratio of materials and in its quality.

Roles played by *Saccharomyces rouxii*. The salt tolerant yeast *Saccharomyces rouxii* strain No. 7[9,10,11], the former name of which was *Zygosaccharomyces major*, grows well in soy mash if it is sufficiently aerated. Although the yeast did not grow satisfactorily because of the insufficient aeration in this experiment, the effects of yeast growth is apparently distinguishable on the table.

The inoculation of the yeast resulted in the consumption of reducing sugars and the production of ethanol. A specific fermentation odour of yeast was not a bad smell in itself, but in this experiment, it was too intense and unfamiliar for soy sauce. The mixed inoculation of both *Ped. soyae* and *Sacch. rouxii*, however, have formed the most satisfactory aroma and flavour almost approximating to those of the normal soy sauce. That is, the yeast fermentation alone can not form the good flavor in soy mash, but the cooperation with *Ped. soyae* is quite necessary.

No other eminent activities of the yeast were observed in this experiment. Acids were not formed, and no influences on *Asp. sojae* enzymes were observed. The decolorizing activity for soy mash exists, but no so strong as that of *Ped. soyae*.

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20) N. Iguchi and K. Yamamoto, *J. Agr. Chem. Soc. Japan*, **26**, 387 (1955).

Growth of *Pediococcus soyae* nov. sp. in Highly Concentrated Solutions of Inorganic Salts and Sugars*

Studies on the Activities of Bacteria in Soy Sauce Brewing. Part VI

By Kenji Sakaguchi

Pediococcus soyae nov. sp., which has an inherited salt tolerant nature, is grown in solutions of high osmotic pressure. When this strain is transferred from 0.5% salted medium to a new medium containing 18% sodium chloride, the viable counts of this organism firstly decrease from about one half to one-third of the inoculated cells, and then normal growth occurs. This indicates the occurrence of physiological adaptation at an early stage of growth.

The growth of this lactic acid bacterium is observed in concentrated solutions of various inorganic salts. The solutions containing Na^+, K^+, Cl^-, NO_3^- and SO_4^{--} ions are not toxic for the organism, and the organism can grow in solutions of 133 atm. osmotic pressure, generally. However, Li^+, Ca^{++}, Mg^{++} and Br^+ are, toxic for growth.

In concentrated sugar solutions, this organism also propagates well, and growth is observed in the media containing 50% glucose or 60% sucrose, osmotic pressure being 105 and 84 atm., respectively. Therefore, *Pediococcus soyae* nov. sp. is osmotolerant.

Pediococcus soyae nov. sp.[1~3], which is a characteristic organism in the brewing procedure of Japanese soy sauce, can grow in a media containing a maximum of 24–26% sodium chloride. This salt tolerancy is an inherited characteristic, and is retained even after pedigree culturing of 31st times in 0.5% salted media.[1]

In this paper, the author reports on the phenomenon of physiological adaptation which is observed on the sudden transferring of 0.5% salt containg culture to 18% salted media. This phenomenon has already been described in the osmophilic yeast *Saccharomyces rouxii*[4] and in the cupper tolerant strains of *Saccharomyces ellipsoideus*[5,6].

The growth in concentrated solutions of various inorganic salts and sugars are also examined and some results are presented in this paper.

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1) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 353 (1958).
2) K. Sakaguchi, *ibid.*, **23**, 22 (1959).
3) K. Sakaguchi, *ibid.*, **23**, 100 (1959).
4) H. Ōnishi, This Bulletin, **21**, 137 (1957).
5) J. Ashida, *Symposium on Cytological Chemistry*, **1**, 111 (1953)
6) T. Minagawa, *ibid.*, **1**, 123 (1953).

EXPERIMENTAL

Basal medium. Usually the following basal medium was used, and various salts and sugars were supplemented for each purpose. Heart infusion broth (Difco) 1%, Polypepton (Takeda) 1%, dipotassium phosphate 0.5%, sodium chloride 0.5% and glucose 1%. The hydrogen ion concentration of the media was adjusted to pH 7.0 after autoclaving, because *Ped. soyae* is acid-sensitive and can only grow between the range from pH 5.5 to 9.0[1]. In order to prepare the agar media, 1.3% powdered agar (Takeda), 0.1% sodium thioglycollate and several drops of methylene blue solution (5%) were added to the broth. Sodium thioglycollate maintains the oxido-reduction potentials of the media low enough permitting growth of *Ped. soyae*, and methylene blue serves as the indicator.

Viable counting of *Ped. soyae* grown in 0.5% and 18% salted media.

Ped. soyae strain *d* 2, which had been stocked in 7% salted agar media, was inoculated in 0.5% salted broth and incubated anaerobically at 30°C for four days.

Five ml of the culture was inoculated to 200 ml in each 0.5 and 18% salted media. They were incubated anaerobically at 30°C, and viable cells grown in the two kinds of broth were counted following the method described below. One ml of the culture was aseptically pipetted and transferred to 50 ml of autoclaved broth, shaked moderately, and 1 ml of the diluted fluid was immediately transferred to the next 50 ml broth. After suitable dilution, 0.5 ml was pipetted, inoculated to 10 ml of melted agar medium and the tube cultured in a high layer. Upon the solidified surface of the agar, was poured uninoculated agar to 2-cm thickness so as to keep the inoculated agar adequately anaerobical. After five to ten day's incubation, colonies grown in the agar were counted.

The culture of 18% salted broth was diluted with the broth of the same salt concentration to avoid osmotic shock, and finally inoculated into two kinds of agar medium containing 0.5% and 18% salt.

RESULTS AND CONCLUSION

Viable cell transition and growth of *Ped. soyae* in 0.5% and 18% salted media.

Ped. soyae has an inherited salt tolerancy, and the upper limit of the salt tolerance is 24–26%. Even in the highly salted media, the fermentation formula of the organism is not changed, proceeding homo-type lactic acid fermentation. The rate of fermentation is however slower[1].

Many drug resistant microorganisms decrease in their viable counts by sudden transfer into the drug added media. When the osmophilic yeast *Saccharomyces rouxii*[4], which is the predominant yeast in soy sauce brewing, is transferred to 18% salted medium from unsalted subculture, only 0.1% to 1% cells gain tolerancy against salt and begin to reproduce, while all the others lose their viability. But the yeast has undoubtedly an inherited salt tolerant nature, and this phenomenon is called physiological adaptation. The cupper tolerance acquistion of *Saccharomyces ellipsoideus*[5,6] is an example of adaptation, and a marked fall of viable cells was noticed during the adaptation phase. *Salmonella pullorum*, *Salmonella schotmülleri* and *Eberthera typhosa*[7] acquired their salt tolerancy through mutation and selection after about one hundred days of incubation. In order to elucidate the adaptation type of *Ped. soyae* by the sudden transfer into highly salted media, the following experiments were conducted.

Ped. soyae strain *d* 2, which had been stock-cultured in 7% salted agar media, was inoculated in a 0.5% salted broth and incubated for four days at 30°C. Five ml of the culture was transferred to two 300 ml conical flasks containing 200 ml of 0.5% salted and 18% salted broth respectively, and incubated at 30°C. The change of viable cell numbers in the two flasks are represented in Fig. 1.

FIG. 1. Growth Curves of *Ped. soyae* in 0.5% and 18% Salted Broth.

—— ○ —— Growth in 0.5% salted broth.
——×—— Growth in 18% salted broth. Colonies counted by 0.5% salted agar.
----△---- Growth in 18% salted broth. Colonies counted by 15% salted agar.

In the 0.5% salted culture, the growth curve of *Ped. soyae* is quite normal, initiating its growth about half a day's lag period, reaching to its maximum counts of 340 million per ml after seven days' incubation, and thereafter the cell numbers decrease rapidly due to the acids produced. However, in the 18% salted broth the viable counts increase in number to about twofold within half a day, thereafter about three-fourth of the cells lose their viability in three days, and then logarithmic growth begins at almost the same speed as usual reaching their maximum counts on the thirteenth day of incubation.

7) J. M. Severens and F. W. Tanner, *J. Bact.*, **49**, 383 (1945).

From these results it is recognizable that in sudden tranferring of *Ped. soyae* from 0.5% salted medium to 18% salted one, the cells show the so-called physiological adaptation as in the case of osmophilic yeast *Saccharomyces rouxii*[4], and reveal the latently inherited salt tolerant character. The ratio of cells which acquire the salt tolerancy is about one half to one-third of the inoculated total cells, and about one-fourth of the one day-cultured cells. This value is much higher compared with *Sacch. rouxii*[4] which decreases its viable counts from 1% to 0.1% of the transferred cells. This phenomenon provides evidence that *Ped. soyae* has an inherited salt tolerant nature.

Fig. 1 also shows that *Ped. soyae* is quite resistant against the osmotic shock of transferring from 18% salted culture to 0.5% salted media. The dotted-curve and the short columned and dotted curve both represent the viable counts of cells in 18% salted culture, but the former counts were counted by the colony numbers grown in 18% salted agar, and the latter in 0.5% salted agar. In any stage of growth, except in the case of the third day, the two curves did not reveal any discrepancy of colony counts. On the contrary, the osmophilic yeasts[4] were very sensitive to osmotic shock, and much decrease in colony counts was observed after the shock.

Growth in media containing a large amount of inorganic salts.

Ped. soyae grows well in media with an extraordinarily high sodium chloride content. The osmotic pressure of the media reaches to 133 atm. at the NaCl concentration of 3 mol (17.6%), and to 187 atm. at the concentration of 4 mol (23.4%)[8] (Table I). The problem whether this

TABLE I. OSMOTIC PRESSURE OF NaCl SOLUTIONS

	Concentration of NaCl	Freezing point depression	Osmotic pressure
1 mol	5.8%	−3.39°C	41.1 atm
2	11.7	−6.93	84.5
3	17.6	−10.89	133.4
4	23.4	−15.11	186.9

8) Landolt-Börnstein, Physikalich-Chemische Tabellen, **2**, 1452 (1923).

TABLE II. GROWTH OF *Ped. soyae* IN MEDIA CONTAINING VARIOUS INORGANIC SALTS

Salts	Osmotic pressure	Lactic acid formed*			
		5 days	8 days	13 days	25 days
NaCl	41.1 atm	1.1 ml	1.6 ml	1.5 ml	1.5 ml
	84.5	1.4	1.4	1.6	1.6
	133.4	0	0	1.4	1.5
	186.9	0	0	0.5	1.5
KCl	41.1	1.0	1.0	1.0	1.0
	84.5	1.0	1.5	1.5	1.4
	133.4	0	1.2	1.5	1.6
	186.9	0	0	0	0
KBr	41.1	1.1	1.1	1.1	1.1
	84.5	0	1.1	1.1	1.1
	133.4	0	0	0	0
	186.9	0	0	0	0
KNO_3	41.1	0.8	1.0	1.0	1.0
	84.5	0.5	1.0	1.2	1.0
	133.4	saturated			
	186.9				
$NaNO_3$	41.1	0.5	0.8	0.8	0.8
	84.5	0.4	0.8	0.8	1.0
	133.4	0	0.5	0.6	1.0
	186.9	0	0	0	0
$MgSO_3$	41.1	0	0	0	0
	84.5	0	0	0	0
	133.4	0	0	0	0
	186.9	0	0	0	0
Na_2SO_4	41.1	1.3	1.4	1.4	1.5
	84.5	1.2	1.6	1.8	2.0
	133.4	0	0	1.7	2.0
	186.9	saturated			
$MgCl_2$	41.1	0	0	0	0
	84.5	0	0	0	0
	133.4	0	0	0	0
	186.9	0	0	0	0
LiCl	41.1	0	0	0	0
	84.5	0	0	0	0
	133.4	0	0	0	0
	186.9	0	0	0	0
NH_4Cl	41.1	0.8	1.0	1.1	1.0
	84.5	—	—	—	—
	133.4	—	—	—	—
	186.9	—	—	—	—

* Values indicate ml of N/20 NaOH consumed in titration of the produced lactic acid in 5 ml culture.

Table III. Growth of *Ped. soyae* in Heavily Sugared Media

	Concentration	Osmotic presure	Titration Value with N/20 NaOH		
			3 days	7 days	10 days
	1%	1.3 atm.	1.40 ml	1.38 ml	1.50 ml
Glucose	20	29.2	1.20	2.25	2.60
	40	74.8	1.40	1.95	2.45
	50	105.0	0.52	1.20	1.45
	60	138.3	0	0	0
	80	204.9	0	0	0
Sucrose	20	19.7	0.75	1.15	1.20
	40	46.7	1.10	1.10	1.25
	50	63.0	0.97	1.37	1.62
	60	84.1	0.44	0.41	0.98
	80	133.5	0	0	0

organism is also tolerant against other inoganic salts or not, namely whether the organism is only salt-tolerant or also osmotolerant, was proposed and the following experiments were carried out. *Ped. soyae.* strain *d* 2 was inoculated in 5 ml of media containing various inorganic salts. The concentrations of these salts were adjusted to the same osmotic pressure with those of media containing 2 mol, 3 mol and 4 mol of sodium chloride, respectively. The inoculated media were incubated anerobically at 30°C and the produced lactic acid was titrated with N/20 sodium hydroxide after five, eight, thirteen and twenty-five days' incubation. The osmotic pressure of various salt solutions were calculated according to Lewis'[9] equation of osmotic pressure and freezing-point depression:

$$\Pi = 12.06\Delta - 0.021\Delta^2$$

In this equation, Π and Δ represent osmotic pressure (atm.) and the degree of freezing-point depression (°C), respectively. With concentrated solutions, the calculated value by this equation fits well with the value actually measured[9]. The degree of freezing-point depression of various salt solutions were taken from Landolt-Börnstein's Physikalisch-Chemische Tabellen[8] and Church's data[10].

The results are given in Table II. Na^+, K^+, Cl^-, NO_3^- and SO_4^{--} ions are not toxic for this organism, and growth is observed even under an osmotic pressure of 133 atm. This value coincides with that of 3 mol or a 17.6% sodium chloride solution.

However, Li^+, Mg^+ and Ca^{++} ions are toxic and the media containing these ions did not permit growth at an osmotic pressure of 41 atm.. Br^- ion had a weak toxicity, inhibiting growth at the osmotic pressure of 84.5 atm. by potassium bromide. This toxic effect however appears to be due to the liberated Br_2 molecule and not to Br^- ion. The medium containing about 2 mol of ammonium chloride changed its pH value from 7.0 to 4.5 as a result of incubation at 30°C in the vacuum jar, which completely inhibited the growth of *Ped. soyae.* The organism could grow under osmotic pressure of 187 atm. only in the solution of sodium chloride.

The above-mentioned facts indicate that *Ped. soyae* is not only salt-tolerant, but is also osmotolerant and grows under the circumstance of 133 atm. osmotic pressure provided the toxic inorganic ions are absent. The toxic ions are almost identical with those for osmophilic yeast *Saccharomyces rouxii*[4].

Growth in media containing large amounts of sugars.

Sacch. rouxii, osmophilic yeast in soy mash, grows well in concentrated sugar solutions as

9) G. N. Lewis, *J. Am. Chem. Soc.*, **30**, 668 (1908).
10) M. B. Church, *Science*, **74**, 492 (1931).

well as in concentrated inorganic salt solutions[4]. It is therefore necessary to examine the growth of *Ped. soyae* in sugared media. The sugars tested were glucose and sucrose, the former is a representative fermentable sugar and the latter is also a representative unfermentacle one for this lactic acid bacterium[1]. The osmotic pressure of glucose solutions were calculated from the data given in International Critical Tables[11], and that of sucrose solutions from the report of Frazer and Myrick[12].

As shown in Table III, growth of *Ped. soyae* could still be observed in the medium containing 50% glucose, of which osmotic pressure of 105 atm. was attained. In the medium containing a large amount of sucrose, growth was observed in a 60% sucrose solution under 84 atm. osmotic pressure.

From these experiments it is concluded that *Ped. soyae* can grow in sugar solutions of about 100 atm. osmotic pressure, if other nutrient substances are sufficient.

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11) International Critical Tables, Vol. 4, p. 429 (1928).

12) J.C.W. Frazen and R.T. Myrick, *J. Am. Chem. Soc.*, **38**, 1907 (1916).

P-Factor, a New Peptidic Growth Factor for *Pediococcus soyae*

Studies on the Activities of Bacteria in Soy Sauce Brewing. Part VII*

By Kenji Sakaguchi

The P-factor a new growth factor for *Ped. soyae*, is found in partial acid and enzymic hydrolysates of Hammersten's milk casein, and is therefore, supposed to be a kind of peptide. The well-known peptidic growth promoting substance for *Lact. casei*, strepogenin and its relating substances, are quite ineffective to the author's organism. P-factor exists along with the S-factor which is another growth promoting-factor for this lactic acid bacterium, in soy mash juice and in the water extract of *Aspergillus sojae* mycelium. The former substance is also found in various kinds of commercial peptones. The factor was not substitutable with all of the 19 synthetic peptides tested. S-factor is effective, only in the presence of the P-factor.

In the former report[1], it was shown that *Pediococcus soyae* nov. sp., which is an important lactic acid bacterium for the brewing of Japanese soy sauce[2], grows only very poorly in a complete synthetic medium for bioassay[3,4], and requires two growth factors tentatively named as P- and S-factors. It was also reported that the P-factor is found in enzymic hydrolysates of Hammersten's milk casein and the S-factor in the heart infusion broth (Difco), yeast extract (Difco) and beef extract (Difco). The S-factor was not substiutable with the following substances; glutamine, asparagine, oleic acid, nyacin amide, pyridoxal, vitamin K_3, choline, inositol, yeast nucleic acid, thymine, ornithine, orotic acid, lipoic acid, mevalonic acid, leucovorin, folic acid and vitamin B_{12}.

The present paper deals with the peptidic nature of P-factor, the difference with the well-known peptidic growth promotant strepogenin[5~17], and also the distribution of P-factor in commercially available peptones, soy mash juices and water extracts of *Aspergillus sojae* mycelium.

EXPERIMENTAL

Assay method.

Two and a half ml of the aqueous solutions to be tested for growth promoting activity were placed in test tubes, and an equal-volume of complete synthetic medium for bioassay[3,4] was then added to each of them. The tubes were plugged with cotton, autoclaved at a pressure of 15 pounds for ten minutes, and cooled. Each tube was then added with 1 drop of a saline suspension of *Pediococcus soyae* strain *d* 2, which had been previously grown for four days anaerobically at 30°C in the following medium: yeast extract (Difco) 0.3%, Polypepton

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1) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 353 (1958).
2) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*, **23**, 100 (1959).
3) G. Tamura et al, *J. Agr. Chem. Soc. Japan*, **26**, 464, 474 (1952).
4) E. E. Snell, *Nutrition Abstr. & Rev.*, **16**, 497 (1946-47).
5) D. W. Wolley and B. L. Hutchings, *J. Bact.*, **39**, 257 (1940).
6) D. W. Wolley, *J. Exp. Med.*, **73**, 487 (1941).
7) N. Grossowiz, *Proc. Soc. Exp. Biol. & Med.*, **49**, 8 (1942).
8) H. Sprince and D. W. Woolley, *J. Exp., Med.*, **80**, 213 (1944); *J. Am. Chem. Soc.*, **67**, 1734 (1945).
9) D. W. Woolley, *J. Biol. Chem.*, **162**, 383; **164**, 11; **166**, 783 (1946); **171**, 443 (1947); **172**, 71 (1948).
10) M. Womack and W. C. Rose, *J. Biol. Chem.*, **162**, 735 (1946).
11) M. L. Scott and L. C. Norris, *ibid.*, **166**, 481 (1946).
12) L. J. Daniel and M. L. Scott., *ibid.*, **174**, 71 (1948).
13) J. L. Stokes, L. K. Koditschek and T. R. Wood, *ibid.*, **178**, 93 (1949).
14) E. Kodicek and S. P. Mistry, *Biochem. J.*, **173**, 479 (1952)
15) D. W. Woolley and R. B. Merrifield, *J. Am. Chem. Soc.*, **76**, 316 (1954).
16) D.W. Woolley, *Arch. Biochem. Biphys.*, **56**, 265 (1955).
17) R. B. Merrifield and D.W. Woolley, *J. Am. Chem. Soc.*, **78** 358 (1956)

(Takeda) 1.0%, glucose 1.0%, dipotassium phosphate 0.5%, sodium acetate trihydrate 2.0% and pH 7.0. The inoculated tubes were anaerobically incubated at 30°C for fifteen days and the produced lactic acid was periodically titrated with N/20 sodium hydroxide. Consequently, it is necessary to run several separate tests to determine exact growth promoting activity of the sample.

Partial hydrolysis of crystalline beef insulin and Hammersten's milk casein with conc. hydrochloric acid.

Merrifield and Woolley[17] reported that high streptogenin activity is observed in the partial acid hydrolysate of crystalline beef insulin. The same hydrolysate was prepared exactly after the description. Crystalline beef insuline (Shimizu Pharm. Co.) (10 mg) plus 0.25 ml of conc. hydrochloric acid were mixed in a tightly glass-stoppered test tube and immediately placed at 37°C and held at that temperature for twenty hours. The hydrochloric acid was removed as completely as possible by distillation under reduced pressure in a water-bath at 37°C. The residue was dried in vacuo over KOH for eighteen hours, dissolved in 1 ml of water, adjusted to pH 7 with NH_4OH.

Hammersten's milk casein (Merck) was also hydrolysed in the same manner. One hundred mg of casein was added with 2.5 ml of conc. HCl and held at 37°C for twenty hours, distilled, dissolved in 10 ml of water and neutralized.

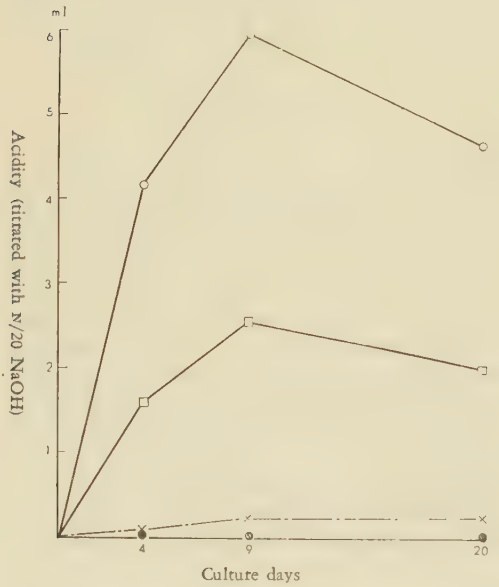

FIG. 1. Dependency of Action of S-factor on P-factor

—●— Synthetic medium
—□— Supplemented with Polypepton 1.0%
—×— Supplemented with beef extract 0.3%
—○— Supplemented with both Polypepton 1.0% and beef extract 0.3%

RESULTS AND DISCUSSION

Dependency of the action of S-factor on P-factor.

Approximately a half of maximum growth of *Ped. soyae* is observed when the basal medium is fortified with P-factor as a partial hydrolysate of milk casein. Further addition of S-factor as Difco beef extract, heart infusion broth or yeast extract results in full growth of the organism[1]. It is questionable however, whether the mere addition of S-factor has any effect on

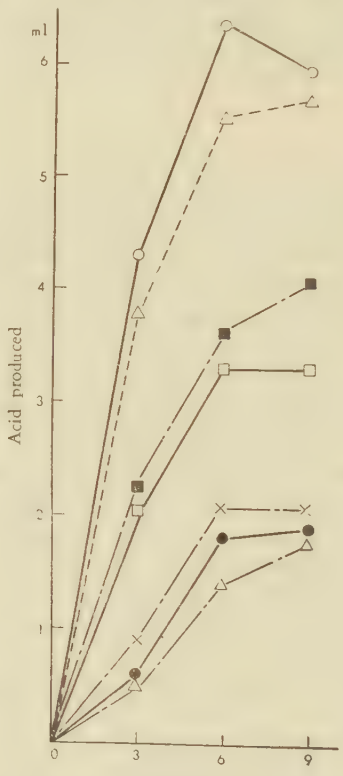

FIG. 2. Effective Concentration of Polypepton

—●— Synthetic medium
—○— Supplemented with heart infusion broth 1.0% and Polypepton 1.0%.
----△---- Suppl. with Yeast extract 0.3% and Polypepton 1.0%.
—■— Suppl. with Polypepton 2.0%.
—□— 1.0%
—×— 0.1%.
---△--- 0.01%.

stimulation of growth of *Ped. soyae*. Therefore, Difco's beef extract which was seemed to be a rich source of S-factor but not of the peptidic P-factor, was added up to 0.3% in the complete synthetic medium for bioassay[1,3,4], and growth curves were determined as shown in Fig. 1. From the curves it is recognized that the addition of both S-factor and P-factor stimulated the growth of *Ped. soyae* strain *d* 2, but the addition of the S-factor alone was almost ineffective for its growth. Therefore, S-factor was effective only in the presence of P-factor.

Effective concentration of Polypeton. The minimum effective concentration of "Polypepton" (Takeda) was examined by adding the substance to concentrations of 2%, 1%, 0.1% and 0.01% into the synthetic medium. The growth curves of *Ped. soyae* strain *d* 2 on these media is shown in Fig. 2. The figure shows that significant growth is not observed below the Polypepton concentration of 0.1%, and a concentration of 1.0% is necessary for full effect of the P-factor.

The figure also indicates the fact that the S-factor can not be substituted with a high concentration of P-factor. Even in the medium which contained 2% Polypepton much less growth was observed as compared with that in the medium which contained both P- and S-factors, namely, supplemented with heart infusion broth (Difco) 1.0% or yeast extract (Difco) 0.3% plus Polypepton (Takeda) 1.0%. Therefore, both the P-factor and S-factor are different substances.

Meanwhile it is worthy to note that a small but measurable growth of the organism was observable in the synthetic medium at this stage (Compare Fig. 2 with Fig. 1). This probably depends on the inoculum size of the organism partly, and also, perhaps on the long stocking of the synthetic medium prepared for two weeks in the refrigerator, which is known to cause some unknown change between antagonistic amino acids[18].

Existence of both P-factor and S-factor in the extract of *Asp. sojae* mold bran and in the unpasteurized soy sauce. To ascertain the existence of the two substances in soy sauce and in soy Koji, *Ped. soyae d* 2 was grown in the synthetic medium supplemented with the unpasteurized soy sauce and with the water extract of molded bran grown with *Aspergillus sojae* strain KS., the soy koji mold[2,19] in use at Noda Shoyu factories. A half-ml of soy sauce which was not yet pasteurized and not added with the antifungal agent (butyl-*p*-hydroxy-benzoate) was added to 4.5 ml of the synthetic medium. The content of the supplemented nitrogenous compounds in the medium was about 1% as protein. The medium supplemented with the water extract of molded bran was prepared as follows: 10 g of molded bran purely cultured with *Asp. sojae* was extracted with 40 ml of distilled water for four hours in the refrigerator, filtered, and 0.5 ml of the filtrate was added to 4.5 ml of the synthetic medium.

As shown in Fig. 3, both the P-factor and S-factor exist in the water extract of molded bran and in the unpasteurized soy sauce. However, it was observed that *Ped. soyae* could not easily grow in soy sauce supplemented with glucose and the pH adjusted at 7.0. This is probably due to many growth inhibitors produced in soy sauce during a maturing[20] period of one year.

Existence of P-factor in several commercial petones. P-factor, which is a peptidic substance produced by an enzymic partial hydrolysis of milk casein, actually exists in "Polypepton" of Takeda Pharmaceutical Co. Ltd.. The existence of this factor in other commercially available peptones is recognized in this experiment. Bacto-peptone, Bacto-tryptone (Difco) or Polypepton (Takeda) were dissolved to 1.0% in the synthetic medium, and growth of *Ped. soyae d* 2 was examined (Fig. 4). P-factor also exists in Difco's two peptones and higher growth responces than in the medium supplemented with Takeda's Polypepton were observed. The reason why higher

18) M.M. Yragui, et al., *J. Bact.*, **69**, 20 (1955).

19) K. Sakaguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 345 (1958).

20) T. Yokotsuka, *J. Agr. Chem. Soc. Japan*, **23**, 200 (1948); **24**, 355, 358, 402, 407 (1951); **25**, 206, 401, 446 (1952): **27**, 276 334, 359, 549 (1953).

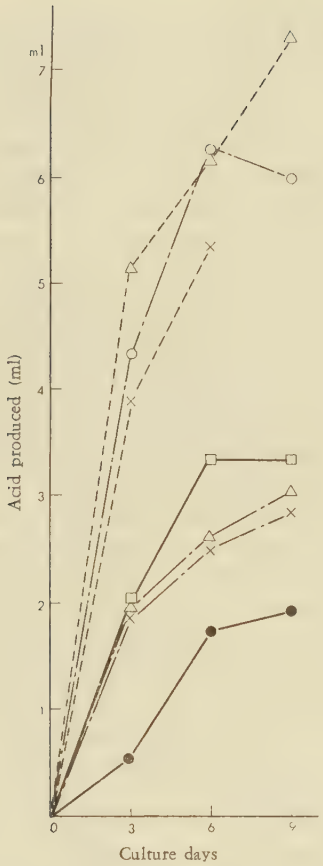

FIG. 3. Existence of P-Factor and S-Factor in Unpasteurized Soy Sauce and in Water Extract of *Aspergillus sojae* Mycelium.

—●— Synthetic medium.
—□— Supplemented with Polypepton 1.0%.
---○--- Suppl. with heart infusion broth 1.0% and Polypepton 1.0%.
----△---- Suppl. with soy sauce (1.0 % as protein)
---△--- (0.1 % as protein)
----×---- Suppl. with the extract of *Aspergillus* mycelium (1 : 4, 0.5 ml).
---×--- (1 : 4, 0.05 ml).

growths are observed on Difco's two peptones will probably be explained by contamination of the S-factor.

Tolerancy of P-factor against attacks of proteinases or HCl. Since the P-factor is assumed to be a kind of peptide, it is conceivable that this substance will lose its biological activity through an attack by various proteinases or hydrochloric acid. Polypepton was hydrolysed with HCl and

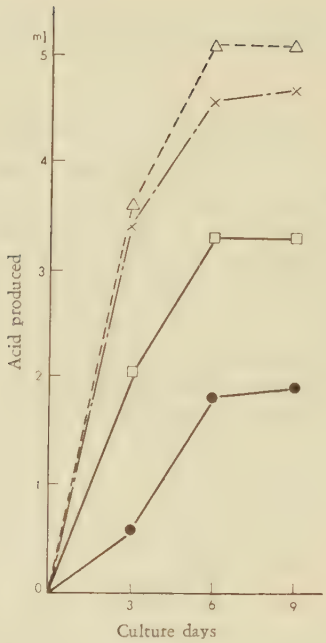

FIG. 4. Existence of P-Factor in Various Commercial Peptones.

—●— Synthetic medium.
—□— Supplemented with Polypepton (Takeda).
----△---- Suppl. with Bacto-peptone (Difco).
---×--- Suppl. with Bacto-tryptone (Difco).

with various proteinases in the following way and their activities on *Ped. soyae* were examined. The proteinases used were; pepsin (Twice crystallized, GBI.), trypsin (Crystalline, GBI.), Nagarse (A crystalline *Bacillus subtilis* proteinase. Nagase Ind. Co.), *Asp. sojae* alkaline proteinase[21,22] (Crystalline, generously supplied by Mr. D. Fukushima of our Institute), *Aspergillus saitoi* acid proteinase[23] (Crystalline, kindly supplied by Dr. F. Yoshida and Mr. M. Nagasawa of our Institute) and *Streptomyces* protease[24,25] G (Crystalline, kindly supplied by Mr. M. Nomoto of the Scientific Research Institute). Among these proteinases, *Streptomyces* protease G which

21) T. Mizunuma and N. Iguchi, *Bull. Agr. Chem. Soc. Japan*, **22**, 35 (1958).
22) W. G. Crewther and F. G. Lennox, *Aust. J. Biol. Sci.*, **6**, 428 (1953).
23) F. Yoshida and M. Nagasawa, *Bull. Agr. Chem. Soc. Japan*, **20**, 252, 257, 262 (1956).
24) M. Nomoto, et al., *Symposia on Enzyme Chemistry* (Japan), **12**, 24 (1957).
25) M. Nomoto, et al., *J. Biochem.*, in press.

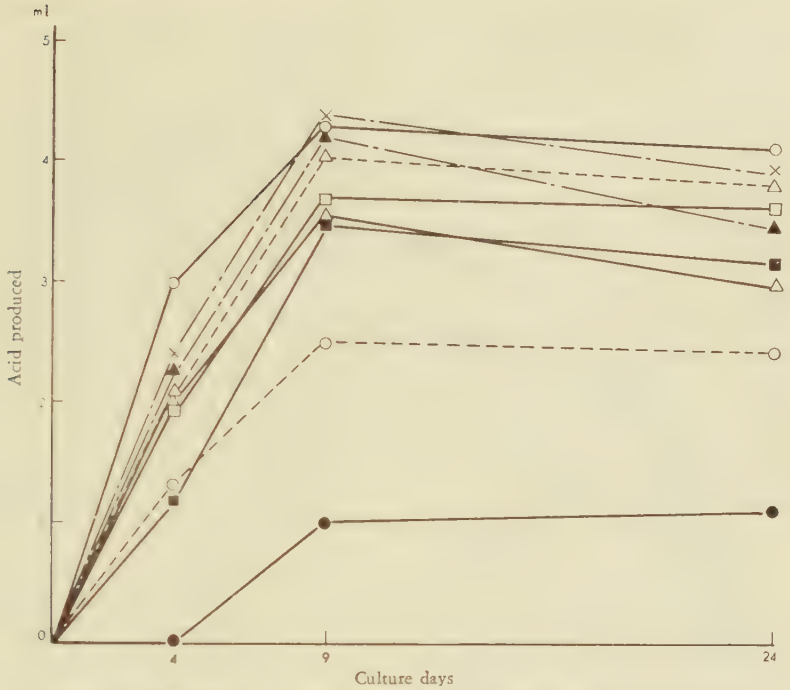

FIG. 5. Digestion of P-Factor with Various Proteinases and with HCl.

is produced in the culture filtrate of *Str. griseus* has the ability to degrade protein molecule more completely than trypsin. This preteinase can digest milk casein perfectly so that under the best conditions, almost 80 to 90% of the constituent amino acids become free. In buffer solutions having the optimal pH value of every enzyme, Polypepton was dissolved to 10% concentration, the enzymes were added at the concentration of 0.05%, and incubated at 37°C for four and a half hours. After incubation, the reaction was stopped by keeping the mixture solutions in a boiling water bath for five minutes and thereafter stored in a refrigerator. The procedure of decomposing Polypepton partially with hydrochloric acid was exactly the same as the method employed in the preparation of strepogenin rich hydrolysate from insulin, which has already been described in this report.

Results are shown in Fig. 5. The P-factor, which is included in Polypepton, has strong resistance against the attack of many proteolytic enzymes and also of cold hydrochloric acid. Its growth stimulation activity for *Ped. soyae* is not diminished after such treatments. Only *Aspergillus sojae* alkaline proteinase succeeded in reducing activity to about half. Especially, the P-factor, was quite tolerant to the attack of trypsin and no decrease of activity was observed after treatment. This is of no wonder, because Polypeton is produced by hydrolysing milk casein exhaustively with an enzyme preparation of which the main component is trypsin.

P-factor activity of various partially hydrolysed

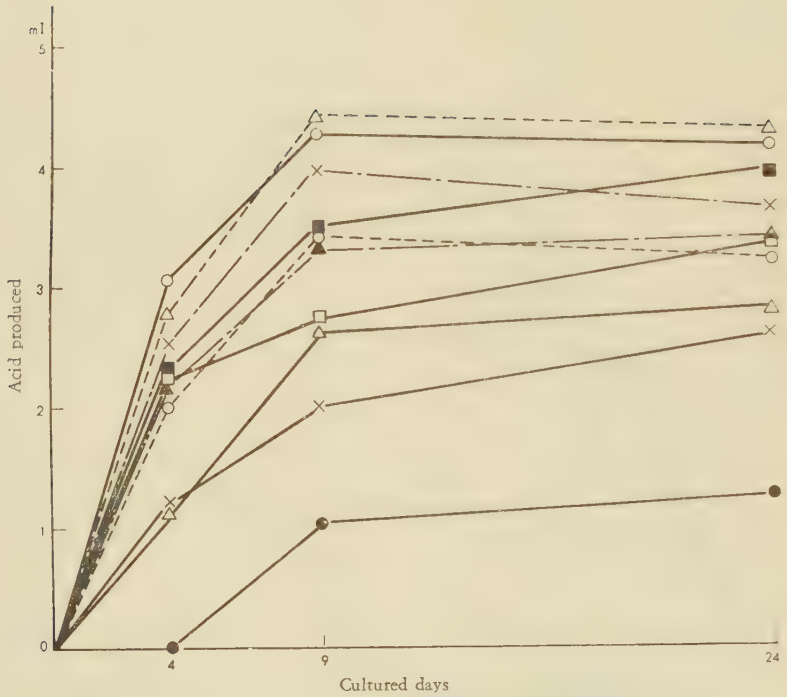

FIG. 6. P-Factor Activity Represented in Various Hydrolysates of Milk Casein.

—●— Synthetic medium.
—○— Supplemented with Polypepton.
—×— Suppl. with unhydrolysed casein.
----△---- Suppl. with hydrolysate by HCl.
—△— Suppl. with hydrol. by pepsin.
---×--- Suppl. with hydrol. by trypsin.
·····○····· Suppl. with hydrol. by *Aspergillus sojae* alkaline proteinase.
—■— Suppl. with hydrol. by *Aspergillus saitoi* acid proteinase.
—□— Suppl. with hydrol. by *Bacillus subtilis* proteinase.
—▲— Suppl. with hydrol. by *Streptomyces* protease G.

milk casein. Fig. 6 shows the growth curves of *Ped. soyae d* 2 which have been inoculated in the synthetic medium supplemented with various enzymic and acid partial hydrolysates of Hammersten's milk casein.

The enzymes and the methods of hydrolysis were identical with those employed in the former experiment. The hydrolysates were added to the synthetic medium at the concentration of 1.0% as protein.

From this figure it is shown that the various enzymic partial hydrolysates of milk casein retain more or less P-factor activity, and also, unhydrolysed whole milk casein somewhat stimulates the growth of the organism. However, the most effective hydrolysate for the lactic acid bacteria is found among those hydrolysates including even Polypepton itself, the one hydrolysed by conc. hydrochloric acid at 37°C for twenty hours. This fact seems to provide evidence that the P-factor is a kind of peptide constituted of the protein molecule of milk casein.

Effect of various synthetic peptides. From the studies on *Leuconostoc mesenteroides*[26] and on other microorganisms it is known that small peptides are generally more nutritious to them than the mere addition of the constitutent amino

26) A. Miller, A. Neidle and H. Waelsch, *Arch. Biochem. Biophys.*, **56**, 11 (1955).

acids. Therefore, it might be said that the peptidic P-factor is not a specific peptide (it may be a group of peptides), but many peptidic molecules are all effective for *Ped. soyae*.

In order to exclude this possibility, various synthetic peptides were collected and their activites were measured by adding them a concentration of 500 γ per ml of the synthetic medium. The collected syntetic peptides were; L-prolyl-L-valine, L-leucyl-L-proline, their anhydrides which are reported by Koaze to be the germination promotants for plant seeds[27], glutathione, glycylglycine, glycyl-L-leucine, L-leucylglycine, L-leucine amide, L-leucyl-glycyl-glycine, L-alanyl-glycyl-glycine, DL-alanyl-DL-aspartic acid, carbobenzoxy-DL-phenylalanine, carbobenzoxy-L-tyrosine, carbobenzoxy-L-glutamyl-L-tyrosine, α-benzoyl-DL-arginine-ethyl-ester, α-benzoyl-DL-arginine-amide, α-benzoyl-glycine-amide and L-chloro-acetyl-L-tyrosine.

As shown in Table I, none of these peptides

Table I. Nutritional Effect of Synthetic Peptides on *Ped. soyae*

Peptides	Acid produced*
L-Pro-L-Val anhydride	0 ml
L-Leu-L-Pro anhydride	0
L-Pro-L-Val	0
L-Pro-L-Leu	0
Gly-Gly	0
Gly-L-Leu	0
L-Leu-NH_2	0
L-Leu-Gly	0
L-Leu-Gly-Gly	0
DL-Ala-DL-Asp	0
DL-Ala-Gly-Gly	0
carbobenzoxy-DL-Ala	0
Cb-DL-Phe	0
Cb-L-Tyr	0
Cb-L-Glu-L-Tyr	0
α-benzoyl-L-Arg-ethylester	0
benzoyl-L-Arg-NH_2	0
benzoyl-Gly-NH_2	0
L-chloroacetyl-L-Tyr	0
glutathione (1 mg/ml)	0

* After 8 days incubation, 5 ml of the cultures were titrated with N/20 NaOH.

could promote the growth of *Ped. soyae*. Since it is reported that glutathione has a weak strepogenin activity for *Lact. casei*, this substance was examined at a concentration as high as 1.0 mg/ml, but no effect was observed. These results show that P-factor activity is not due to unspecific peptides, but is due to a group of specific peptides which are biologically active.

Difference of P-factor from strepogenin. Strepogenin is well known[5~17] as for the peptidic growth promotant for lactic acid bacteria. The substance, which stimulates the growth of haemolytic *Streptococci*, *Lactobacillus casei* and Smith's *Streptococcus lactis*, is included in acidic and enzymic partial hydrolysates of various protein, especially insulin and milk casein. It is not a single peptide, and the identified several active peptides are all different in their molecular size and their activities[9,15,17]. Among these the most effective are: the pentapeptide seryl-histydyl-leucyl-valyl-glutamic acid and the heptapeptide seryl-histydyl-leucyl-valyl-glutamyl-alanyl-leucine which are isolated from the acid hydrolysate of insuline[17]. The pentapeptide was synthesized by Prof. Akabori and his collaborators at the University of Osaka, and this substance (100 γ/ml) was tested for the P-factor activity. Two peptidic hormones of the posterior lobe of pituitary body, vasopressin[28] and oxytocin[29], are known to have high potencies of strepogenin activity[15], and these substances were also tested. Two International Units of synthesized oxytocin (Sandoz Co. Ltd.. Labelled as Syntocinon.) were added to 5 ml of the synthetic medium for lactic acid bacteria, autoclaved at 115°C for ten minutes, and inoculated with the washed supension of *Ped. soyae d* 2. The vasopressin preparation used here is an injection of Parke, Davis & Co., named Pitressin, which is not a synthetic drug, but a fractional extract of the posterior pituitary gland, and can not be considered to be a pure substance. Moreover, it was necessary to remove the added preservative chlorobutanol by extraction with

27) Y. Koaze, *Bull. Agr. Chem. Soc. Japan*, **21**, 197 (1957); **22**, 91, 98, 238 (1958).

28) V. du Vigneaud, et al., *J. Am. Chem. Soc.*, **75**, 4880 (1953).

29) V. du Vigneaud, et al., *ibid.*, **75**, 4879 (1953).

ether. After pretreatment, 4 International Units of the subtance were examined as usual. The partial acid hydrolysate of insulin is reported by Merrifield and Wolley[17] to be rich of strepogenin activity, and 10 mg of the hydrolysate was added into the synthetic medium.

TABLE II. NUTRITIONAL EFFECT OF STREPOGENIN AND ITS RELATING SUBSTNCES ON *Ped. soyae*

Peptides	Concentration	Acid produced
Ser-His-Leu-Val-Glu	100 γ/ml	0 ml
partial hydrolysate of insulin	2 mg/ml	0
vasopressin	4 units/ml	0
oxytocin	0.4 units/ml	0

Table II indicates that all strepogenin and its relating substances are ineffective to stimulate the growth of *Ped. soyae*. It is thus affirmable that P-factor is a series of biologically active peptidic substances different from strepogenin*.

The author wishes to express his hearty thanks to Prof. T. Asai and Prof. K. Kitahara of the Institute of Applied Microbiology, Univerty of Tokyo for their kind guidance. Thanks are also due to Dr. G. Tamura, Dr. A. Obayashi, Mr. Y. Koaze of the University of Tokyo and Mr. J. Kirimura of the Sericulture Experiment Station for their generous supply of precious growth promotants. The author is greatly indebted to Dr. M. Mogi, and members of this Institute for their kind advice and to Mr. T. Kuramochi for his willing and able technical assistance.

* Recently, this conclusion was affirmed by D. W. Woolley in his private letter.

Studies on Koji*

Part IV. Mold Protease Production by Replacement Culture and Inactive Form Protease in Cell Free Extract

By Kisirō Yamamoto

The effects of growth temperature on protease producing abilities of washed mycelial cells of *Aspergillus soyae* KS were investigated. The maximum ability of the cells grown at 35°C was almost the same as that of the cells grown at 20°C. Cells of 35°C culture rapidly lost their protease producing abilities after it reached its maximum, whilst cells of 20°C culture maintained their abilities for a comparatively long time until a later cultural time.

Effects of temperature on protease production of washed cells were the same as those obtained with growing cells. It was recognized that the protease activity of cell free extracts of mycelia increased during incubation. Thus it was supposed that there would be a zymogen precursor in the process of protease formation by this mold.

It has been reported that[1] the lower the cultural temperature of *Asp. soyae* KS, the larger is the amount of protease produced. In order to confirm this point, the increase of protease activity in washed cells from two growing cultures at 20°C and 35°C was compared. Sioda et al.[2] have shown that when washed cells of *Asp. oryzae* A–1–5, which scarcely produced protease in the growth medium, were shaken in water or phosphate buffer, a considerable amount of protease is produced. They suggested that[3] this condition would have forced mold cells to an abnormal biosynthesis[4,5] which competes with the normal protein synthesis, and protease would be produced by such an abnormal biosynthesis. Accordingly, it seemed possible to clarify the protease productivity of mycelia of growing cultures by comparing the increase in protease of washed cells during incubation under the same conditions with the initial culture. Subsequently, it is also found in this paper that when cell free extracts (C.F.E.) of *Asp. soyae* KS are incubated at various temperatures, the protease activity in the extracts are increased. This phenomenon was recognized only with the extracts of mycelia which were separated from the growing culture, activity producing much protease. From the results of experiments using C.F.E., it seemed that this mold must have a zymogen precursor as an intermediate in the process of protease formation. These results will be also reported here.

EXPERIMENTAL

1. Strain used *Asp. soyae* KS was chosen as mentioned in the previous paper[1].

2. Culture Media Medium for paste culture was prepared by the same method as descibed in the previous report[6]. Four kinds of media were chosen for liquid culture as shown in Table I[2,7,8], and a quantitiy

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1) K. Yamamoto, *Bull. Agr. Chem. Soc. Japan*, **21**, 319 (1957).
2) H. Sioda and K. Sakaguchi, *J. Agr. Chem. Soc. Japan*, **23**, 426 (1950).
3) H. Sioda, *J. Agr. Chem. Soc. Japan*, **30**, 724 (1956).
4) A. Matsushito, T. Miwatani and D. Nakada, Biological Science (Tokyo) p. 7 (1956).
5) M. Nomura and J. Hosoda, *J. Agr. Chem. Soc. Japan*, **30**, 233 (1956).
6) K. Yamamoto, *Bull. Agr. Chem. Soc. Japan*, **21**, 308 (1957).

TABLE I. COMPOSITION OF GROWTH MEDIA

component	Kind of Medium (g/l) A	B	C		D	
glucose	10	50	sucrose	30	sucrose	40
peptone	4.2	30	$NaNO_3$	2	NH_4Cl	11
KH_2PO_4	30	5	K_2HPO_4	1	K_2HPO_4	0.4
$MgSO_4$	0.1	2.5		0.5		0.4
$ZnCl_2$,$FeSO_4$	trace	trace	trace		trace	
NaOH	7				Na,K-tartrate	30

A. B and D medium are used in experiments of mold protease formation by K. Motonaga[7], H. Sioda et al.[2] and M. E. Maxwell[8], respectively, and C shows Czapek-Dox's medium.

of 150 cc of each liquid medium was taken into 500 cc flasks, and it was sterilized for 15 minutes under a steam pressure of 10 lb.

3. Replacement Culture The mycelial cells obtained from each culture were washed several times with distilled water, pressed with a filter paper, and placed in a double petri-dish not being brought into contact with any solution. The outside space of this double petri-dish was filled with distilled water in order to prevent the drying of washed mold cells. Then, the double petri-dishes containing the washed cells were kept at 35°C or 20°C.

4. Enzyme Preparation Appropriate amounts (20–60 mg) of the mycelial cells from paste culture, surface culture, or replacement culture, were mashed well in a glass-mortor with 20 cc of phosphate buffer (pH 7.0, M/15) and glass sand. After keeping the mashed cells for 4 hours at room temperature, the filtrate was taken and used as the enzymes preparation of each culture.

5. Determination of Protease Activity Protease activity of each preparation was measured in the same way as described in the previous report[1].

6. Cell Free Extracts and Determination of its Protease Activity The washed growing cells were pressed with a layer cloth to remove water and were mashed in a glass-mortor with the same volume of glass sand, and the homogenates were then extracted with two volumes of phosphate buffer (pH 7.0, M/15). The supernatant, being separated from the cell debris with freezing centrifugation (0°C, 10^5 r.p.m., 10 mins), was used as the cell free extracts.

Another preparation which was extracted from aceton-dried cells with 10 volumes of phosphate buffer was also used. All processes of C. F. E. preparation were carried out at 0°C. The C. F. E. was used immediately after preparation, and the protease activity in C. F. E. was determined at intervals by the usual method.

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RESULTS

1. Increase of Protease Activity by Replacement Culture Method. Mycelial cells from paste cultures at 35°C and 20°C were washed well, and then these mycelia free from culture media were kept at 20°C and not brought in contact with any media. Fig. 1 shows that the protease activities in washed cells were increased, accompanying a definite increase in the spore number. It is also shown that any cell from cultures of various phase could attain its maximum protease activity within almost 48 hours' incubation after replacement.

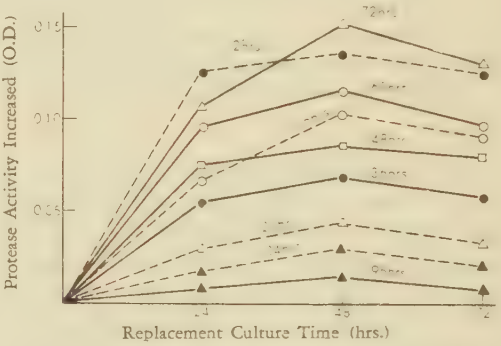

FIG. 1. Increase of Protease Activity in Washed Cell by Replacement Culture.

----- Cells from 35°C culture
——— Cells from 20°C culture

Figures (hrs) on the curves show a time of culture phase of washed cells.

Replacement culture was carried out under the condition which washed cells were kept at 20°C without touching any media: being common to Figs. 1, 2 and Table III.

2. Comparison of Protease Productivity between the two Mycelial Crops grown at 35°C and 20°C. The protease activity per mg of dried mycelial weight which increased during replacement culture (48 hrs, at 20°C) was used as a measures of protease productivity of the mycelium. As shown in Fig. 2, the protease producing ability of mycelium of 35°C culture taken at a certain phase of growth was equal to that of 20°C, that is, there was no significant difference be-

tween the maximum values of protease producing ability of mycelia from 35°C and those of mycelia from 20°C culture. However, a marked difference was observed in the protease producing ability of mycelium from the 35°C culture which decreased rapidly after its maximum, while the mycelium from the 20°C culture maintained such a high level of protease productivity until its later phase of growth. Moreover, the weight of mycelium from the 20°C culture was heavier than that from 35°C culture, when compared at the time producing the maximum level of protease (Fig. 2).

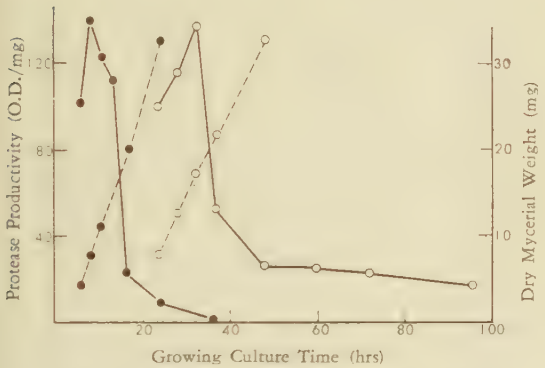

FIG. 2. Protease Productivities and Dry Weight of Washed Cells from 35°C and 20°C Culture.

—●— 35°C culture Protease productivities
—○— 20°C culture ----- Dry mycelial weight

The protease activity increased during replacement culture (48 hrs, at 20°C) per mg of dry mycelial weight was used as a measure of protease productivity of the washed cells.

3. Effect of the Temperature of Replacement Culture on Protease Production. The protease production of mycelia, which were harvested from the same growing culture and were secondary cultured in petri-dishes at different temperatures, was compared. Fig. 3 shows that protease production at 35°C was more rapid than that at 20°C, but after showing maximum activity the decrease in protease in the mycelium at 35°C was more rapid than that at 20°C. The maximum protease activity in the mycelia incubated at 35°C was lower than that at 20°C. These results with regard to the effect of the

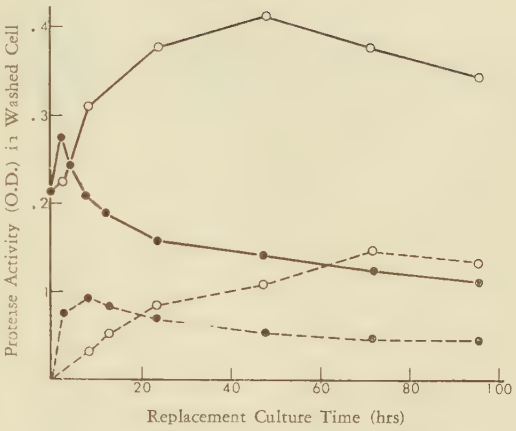

FIG. 3. Effect of Temperature of Replacement Culture on the Protease Production of Washed Cell.

—●— 35°C
—○— 20°C
------- Cells from 35°C culture at 12 hrs. of growth phase
——— Cells from 20°C culture at 72 hrs. of growth phase

temperature of replacement culture were the same as those obtained with growing culture described in a previous report[1].

4. Increase of Protease Activity in Cell Free Extracts. The increase in protease activities of the cell free extracts by incubation was next investigated. The extracts were prepared from the mycelial cells which were grown on the four different media. From the results shown in Table II, it can be said that the protease activities of the extracts from mycelia which produced much protease in growth media were markedly increased during incubation, but the extracts from mycelia which produced little or no protease did not show such an increase. The protease productivities in replacement culture of the mycelia which have grown on mediums A and B are shown in Table III. It can easily be pointed out that the C.F.E. of mycelia grown on B medium at 24 hours of culture did not have the ability to increase protease during incubation, though the washed mycelia could produce a considerable amount of protease in replacement culture without added

Table II. Increase of Protease Activity in Cell Free Extracts from Various Growing Cells

kind of medium	cultural time (hrs)	pH of medium	mycelial weight (g)	protease in medium (O.D./g)	protease in mycelium	protease increased in C. F. E.
A	24	7.0	0.9	0.05	0.03	0.03
	48	7.0	2.0	0.090	0.02	0.03
	72	7.0	2.1	0.110	0.015	0.020
	96	7.0	2.0	0.110	0.010	0.015
B	24	5.0	0.8	0	0.010	0
	48	4.9	1.5	0	0.015	0
	72	4.8	3.0	0	0.020	0.040
	96	5.9	8.0	0.002	0.030	0.130
	120	7.6	9.0	0.010	0.060	0.080
C	48	6.6	1.5	0	0.005	0
	72	5.7	2.4	0	0.010	0
	96	6.5	3.0	0	0.010	0
D	24	5.4	1.0	0	0	0
	48	5.3	3.0	0.005	0.005	0.010
	72	5.6	4.0	0.005	0.020	0.030
	96	5.8	4.0	0.010	0.015	0.015

Values indicate optical density as protease activity: being common to Table II–VI.

Table III. Increase of Protease Activity of Washed Cells from two Cultures

medium	cultural time (hrs)	protease in medium	protease in mycelium	protease increased
A	24	0.050	0.067	0.134
	48	0.100	0.061	0.066
B	24	0	0	0.129
	48	0	0.021	0.030

Table IV. Effect of Potato-inhibitor on the Protease Activity Increased in C. F. E.

	incubation time (mins)		
potato-inhibitor	0	90	180
none	0.030	0.072	0.156
added	0.015	0.013	0.010

Potato-inhibitor was prepared by Yasui's method. The pre-incubation was performed for 5 minutes at 30°C.

nutrients.

5. Some Characteristics of Increase in Protease Activity in C.F.E.

a) Kind of protease increased: It is now recognized that *Asp. soyae* KS produces three kinds of proteinase: alkaline, neutral and acid proteinase[9], and it is well known that the potato-inhibitor specifically inhibits the activity of alkaline protease[10,11]. By the addition of potato-inhibitor to C.F.E., it was proved that alkaline protease is the main component of protease which increased during incubation of C.F.E. (Table IV).

b) Effect of incubation temperature: The increase of protease activity in C.F.E. at various incubation temperatures was observed. It was found that C.F.E. could reach its maximum protease activity within 4 hours at 30°C and 48 hours at 0°C during incubation at pH 7.0 (Fig. IV).

c) Effects of inhibitors, ATP and amino acid mixture: The effect of the addition of inhibitors of protein synthesis, ATP or an amino acid mixture on the increase of protease activity in C.F.E. were then examined. These substances were found to have no effects on the increase of protease activity in C.F.E. (Table V).

d) Effects of C.F.E. on alkaline protease: It was investigated whether there was any change in the activity of alkaline protease when C.F.E. was added to alkaline protease solution.

9) K. Yamamoto, *Bull. Agr. Chem. Soc. Japan*, **21**, 313 (1957).
10) K. Matsushima, *J. Agr. Ghem. Soc. Japan*, **29**, 883 (1955).
11) T. Yasui, Lecture delivered at the meeting of J. Agr. Chem. Soc. Japan (1955).

The results showed that there was no difference between the protease activities with and without the addition of C.F.E. (Table VI).

e) Activities of C.F.E. with various concentrations of casein: The course of hydrolysis of casein by C.F.E. was examined (Fig. V) and it was found that C.F.E. hydrolysis casein of various concentrations show the same hydrolysis-curve.

DISCUSSION

From the results obtained with washed mycelial cells, the presumption that the mycelium which had been cultured at lower temperature might have much stronger ability for protease production than that cultured at higher temperature was excluded. Both the mycelia from the 35°C culture and those from 20°C culture showed the same degree of ability to produce protease, when compared at certain phases of growth (Fig. 2). However, the total amount of protease produced was larger in the mycelia grown at lower temperatures.

Another difference between the 35°C culture and 20°C culture was that the protease productivity of the mycelia from 20°C lasted

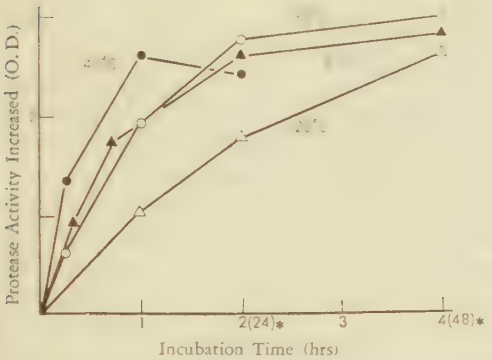

FIG. 4. Effect of Incubation Temperature on the Increase of Protease in C. F. E..

* Shows the incubation time (hrs) for the incubation at 0°C.

Cell free extracts were incubated at various temperature and at pH 7.0.

TABLE V. EFFECT OF SOME SUBSTANCES ON THE INCREASE OF PROTEASE ACTIVITY IN C. F. E.

substances added		relative activity of increased protease (%)
none		100
2, 4-DNP	final conc of 10^{-3} M	112
8-oxy-quinoline	//	82
chrolum phenycohl	//	103
NaN_3	//	96
Ethionine	//	103
8-aza-guanine	//	109
penicillin, aureomycin, streptomycin	//	103
amino acid mixture	//	101
A. T. P.	//	105

The cell free extract was incubated for 3 hours (pH 7.0, 30°C), after being mixed with each substance.

TABLE VI. EFFECT OF C. F. E. ON ALKALINE PROTEASE

composition of mixture (total volume of 10 cc)		protease activity measured	protease activity calculated	differentiation
C.F.E.	alk. protease			
5	5	0.226	0.230	−0.004
2.5	5	0.192	0.190	+0.002
5	0	0.070		
2.5	0	0.030		
0	5	0.160		

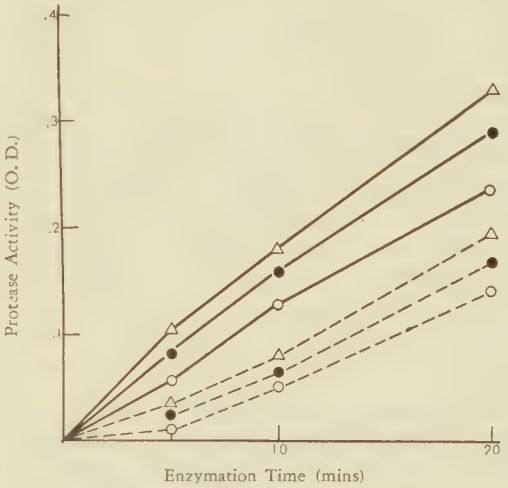

FIG. 5. Courses of Hydrolysis by C.F.E. and by Alkaline Protease on Various Concentration of Casein.

longer than that of the mycelia from 35°C. These facts might be attributed to the differences in the dry weight (mg) of mycelial cells: the dry weight of mycelia grown at 20°C is much larger than those grown at 35°C. In this paper, the increased protease activities during replacement culture were used as a measure of protease productivity. Then arises the question—what is the source of this increased protease in mycelial cells? Two possibilities are suggested: (1) the mycelia contain the precursor or (2) protease is newly synthesized by the cells upon incubation. The presence of zymogen precursors of protease is well known in animal bodies. Recently, zymogen precursors of Streptococcal proteinase and of Coccus P proteinase were also discovered by S.D. Elliott et al.[12], and L. Gorini et al.[13], respectively. Nomura et al.[14], and Fukumoto et al.[15] have also assumed the presence of some precursors in the process of amylase formation by *Bac. subtilis*. Working with *Asp. Kawachii*, Kanie[16,17] recognized the increase of amylase activity in C.F.E. and presumed the presence of a precursor of amylase in mycelial cells. However, no study has been made concerning the presence of the precursor of koji mold protease. From the results obtained in the present paper, it is concluded that there must be zymogen precursor in the process of mold protease formation.

That the protease is *de nove* synthesis of protein would not be probable, because the protease production was not inhibited by the addition of inhibitors of protein synthesis nor by the addition of metabolite analogues such as ethionine and 8-azaguanine (Table V). Since the mode of hydrolysis of casein by C.F.E. did not vary with increasing casein concentration (Fig. 5), it is not plausible that protease molecules in the C.F.E. are already conbined with some substrates which compete with casein. On the other hand, examination of the effects of incubation temperature on the mode of hydrolysis of casein by C.F.E. appeared to indicate that some kind of enzyme action is involved in the increase of protease activity of C.F.E. (Fig. 4). Thus, it seems that the protease activity increase in C.F.E. during incubation resulted from activation of the zymogen precursor which was contained in C.F.E.. As shown in Table II, all the C.F.E. prepared with mycelial cells which had been actively producing protease in growth media showed the same extent of increase in protease activity upon incubation. This would suggest that the zymogen precursor would not be stored in the mycelial cells during growth, but it is immediately transformed to the active enzyme as soon as it appears. On the other hand, the fact that the mycelial cells grown for 24 hours in medium B produced protease during replacement culture whereas incubation of C.F.E. of the same cells did not result in any increase in protease activity seems to show that the increased protease in the replacement culture was not attributable to zymogen precursor but was newly synthesized (Table II, III).

With regard to the zymogen precursor and its activating enzyme (kinase), further investigations are now in progress.

The author wishes to express his sincere thanks to Prof. K. Sakaguchi for his kind suggestions rendered throughout the course of this study, and to Dr. M. Nomura and Dr. F. Yoshida for their advice.

The author also wishes to thank Dr. M. Mogi for kindly making many facilities available for the purpose of this study.

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Studies on Osmophilic Yeasts*

Part IV. Change in Permeability of Cell Membranes of the Osmophilic Yeasts and Maintenance of their Viability in the Saline Medium

By Hiroshi Ōnishi

A striking increase in permeability of cell membranes of *Saccharomyces rouxii*, a typical salt-tolerant yeast, occurred when they were cultivated in the saline medium. This change in permeability, however, did not occur in the concentrated sugar solution. It appeared that this increase in permeability should be attributed to the effect of sodium chloride itself, rather than to the osmotic effect. Therefore, this increase in permeability must be one of the most important points that serve to elucidate the mechanism of salt-tolerant property of yeast cells. The cells which had been changed in permeability by the effect of sodium chloride, could not maintain their viability in the saline medium unlees an active metabolic process was allowed to proceed with the aid of the external substrates.

INTRODUCTION

In the previous paper[1], in which the nature of salt-tolerance and that of sugar-tolerance of the osmophilic yeasts were compared, it was found that the salt-tolerance and the sugar-tolerance are quite different. It therefore seemed that some factors other than the osmotic pressure must be involved in the salt-tolerant mechanism of the osmophilic yeasts. Supposing that mechanism of their salt-tolerance might mainly depend upon the environmental influence of the ions or the undissociated molecule of sodium chloride, the change of sodium and potassium contents of the cells in the saline medium was first observed in the present paper. A remarkable increase in permeability of cell membrances was found to occur in the saline medium, and the significance of this phenomenon in the salt-tolerant property of yeast cells is discussed.

EXPERIMENTAL AND RESULTS

[I] Increase in Permeability of Cell Membranes in the Saline Medium.

Although important roles of permeability of cell membranes in the salt-tolerant and halophilic microorganisms are naturally expected[2], very few investigations have hitherto been conducted from this view point. Conway et al.[3] prepared a "Na-yeast" in which all or nearly all the cellular potassium could be replaced by sodium, and the role of potassium and sodium in yeast physiology was investigated by comparing the property of such a yeast with those of the control potassium yeast. The mechanism of transport of potassium and sodium through cell membranes was discussed by Conway[4]. Takada[5] reported that a salt-tolerant mutant of *Saccharomyces ellipsoideus* showed an increased permeability to sodium as compared with the parent strain. Since studies on the characteristics of salt-tolerance of osmophilic yeasts in a high concentration of sodium chloride such as 18 percent have not heen

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Table I. Contents of Potassum and Sodium of *Sacch. rouxii* Cultivated in the Concentrated Solutions of NaCl or Glucose

culture media	washing solutions	yeast dry matter 1 g	
		K (mg)	Na (mg)
ordinary koji extract (bllg. 8)	distilled water	10.38	0.57
koji extract containing 18% NaCl	distilled water	0	0.47
	5% glucose water	0	0.47
	50% glucose water	0.57	0.94
	5% glucose 18% NaCl water	3.44	—
	5% glucose 18% KCl water	—	1.43
koji extract containing 50% glucose	distilled water	9.92	0.52

carried out from the standpoint of membrane-permeability to potassium and sodium ions, some experiments were conducted using *Saccharomyces rouxii* which is a typical salt-tolerant yeast[6] and is known to play an important role in soy-brewing.

[1] Potassium and Sodium Contents of *Saccharomyces rouxii* and the Change in Permeability of Cell Membranes when Cultivated in the Concentrated Solutions of Sodium Chloride or Sugars.

Method: Cells of *Sacch. rouxii* cultivated at 30° for 7~13 days in koji extract containing NaCl 0%, 18% or glucose 50%, were repeatedly centrifuged and washed three times with various washing solutions. The washed yeast cells were digested by boiling with conc. nitric acid, and the resulted solution was evaporated to a minimum volume to remove nitric acid and then diluted to an appropriate amount with distilled water. Analysis of potassium and sodium was carried out employing a Perkin-Elmer C-52 Flamephotometer. The results are shown in Table I.

It is very striking fact that yeast cells cultivated in the koji extract containing 18% sodium chloride showed a remarkable increase in permeability of potassium; almost all of the potassium in the yeast cells were lost by washing with distilled water. However, this complete loss of potassium in the cells did not appear to occur during growth in the high saline medium, because a considerable amount of potassium was still retained within the cells when they were washed with the solutions of the same osmotic pressure as that of 18% NaCl solution. The content of sodium in the cells remained unchanged in this washing, indicating that sodium ions do not enter into the cells in place of the extruded potassium. When these cells were washed with a 5% glucose solution, which should serve as the energy source to this yeast, potassium was washed out similarly as in the case of washing with distilled water. On the contrary, this change in permeability of cell membranes could not be recognized in the cells grown in the 50% glucose medium, which had the same osmotic pressure as that of 18% NaCl.

(2) Change in Permeability when Yeast Cells grown in the Ordinary Culture Medium were Suspended in a Phosphate Buffer Containing 18% NaCl.

Method: Cells of *Sacch. rouxii* cultivated in ordinary koji-extract at 30° for 7 days were washed twice with distilled water by centrifugation, and the washed cells were then suspended in the M/15 phosphate buffer solution (pH 4.8) containing 18% NaCl with or without the added glucose, and the suspensions were incubated at 30°. Cells taken at each interval were washed and analysed for potassium and sodium as similarly as described in (1). The results are shown in Table II.

From Table II, it is clear that the increase in permeability of the cells without the process of cell multiplication could be fully observed when the cells of the oridnary culture were suspended in the 18% NaCl phosphate buffer for 24 hours at 30°, and in this case any significant difference in the process of permeability change could not be recognized between the two suspending media with and without added glucose (5%).

However, a significant difference was found in the viability of the yeast cells between the above two environments with and without glucose; a remarkable loss in viability occurred in the glucose-free 18% NaCl phosphate buffer whereas, in the 18% NaCl phosphate buffer with added glucose (5%) no loss or only a slight

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TABLE II. CHANGE IN PERMEABILITY WHEN THE CELLS OF THE ORDINARY CULTURE WERE SUSPENDED IN THE 18% NaCl PHOSPHATE BUFFER

period in suspending (hrs.)	glucose-free 18% NaCl phosphate buffer yeast dry matter 1 g				5% glucose 18% NaCl phosphate buffer yeast dry matter 1 g			
	K (mg)		Na (mg)		K (mg)		Na (mg)	
	washed by dist. water	washed by 18% NaCl water	washed by dist. water	washed by 18% KCl water	washed by dist. water	washed by 18% NaCl water	washed by dist. water	washed by 18% KCl water
0	11.2	—	0.3	—	11.2	—	0.3	—
1	3.9	—	1.3	—	3.6	—	1.5	—
3	3.6	—	1.6	—	3.6	—	1.6	—
24	0	3.4	1.6	—	0	3.8	0.6	2.6
48	0	1.0	0.7	2.7	0	2.6	0.4	1.9

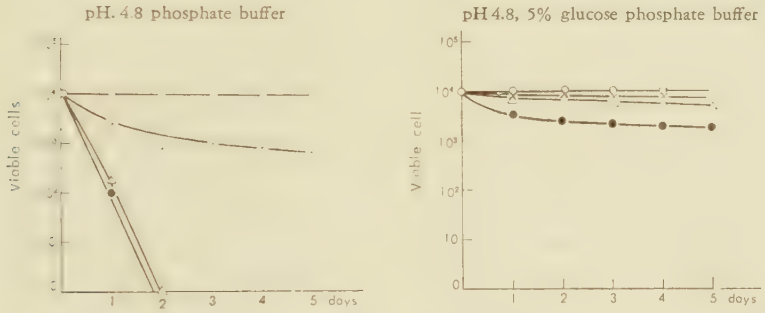

FIG. 1. Effect of Glucose on the Maintenance of Viability in the Concentrated Solution of NaCl (1).

(When the cells of the ordinary culture were transferred to the saline medium)

○: NaCl 0, ×: NaCl 1 mol, △: NaCl 2 mol, ●: NaCl 3 mol.

loss of viability took place. (Fig. 1).

When cells of the ordinary culture were suspended in the phosphate buffer containing sodium chloride of a relatively low concentration such as 5%, the increase in cell permeability was also recognized as shown in Table III.

TABLE III. CHANGE IN PERMEABILITY WHEN THE CELLS OF THE ORDINARY CULTURE WERE SUSPENDED IN THE 5% NaCl PHOSPHATE BUFFER

washing solutions	yeast dry matter 1 g	
	K (mg)	Na (mg)
distilled water	0.63	1.10
5% glucose 5% NaCl water	5.51	—
5% glucose 5% KCl water	—	1.49

(3) Effect of Temperature on Change in Permeability of Yeast Cells in the Saline Medium.

When the cells of *Sacch. rouxii*, cultivated in koji extract, were suspended in the 18% NaCl phosphate buffer with or without glucose and kept in a refrigerator (at 0°～5°), any change in permeability could not be observed even after a long period of storage, unlike those in the case of 30°C. This phenomenon was similarly found to occur without regard to the addition of glucose. The results are shown in Table IV.

In this case, it was also noted that viability of the cells of the 18% NaCl medium was maintained without the addition of glucose to the suspending medium (Fig. 2).

It was further observed that loss in viability rapidly occurred when the cells of *Sacch. rouxii*, cultivated in the 18% NaCl medium, were transferred to the glucose-free 18% NaCl phosphate buffer, though the loss of viability of cells was tolerably prevented by the supply

TABLE IV. EFFECT OF THE LOW TEMPERATURES ON CHANGE IN PERMEABILITY OF *Sacch. rouxii* IN THE SALINE MEDIUM

period in suspending (days)	glucose-free 18% NaCl phosphate buffer yeast dry matter 1 g		5% glucose 18% NaCl phosphate buffer yeast dry matter 1 g	
	K (mg)	Na (mg)	K (mg)	Na (mg)
0	10.61	0.66	10.61	0.66
1	5.60	3.76	5.60	3.47
5	2.13	3.54	1.76	3.24

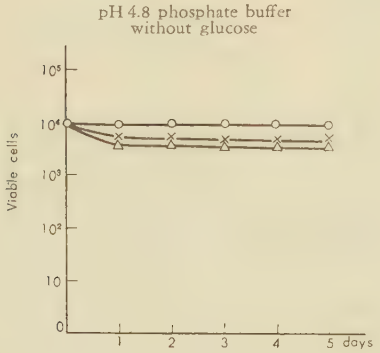

FIG. 2. The Maintenance of Viabitity in the Saline Medium at the Low Temperature of 0°～5°.

○: NaCl 0%, ×: NaCl 10%, △: NaCl 18%.

of glucose. (Fig. 3).

From the above facts, it was presumable that the viability of *Sacch. rouxii* in the concentrated solutions of sodium chloride was maintained without any supply of glucose under the conditions in which no increase in cell permeability could occur, but no matter how sufficiently they might be adapted toward sodium chloride, the cells, which had an increased cell permeability in the saline medium, would need some active metabolism by utilizing the energy source such as glucose for the maintenance of their viability in the concentrated solution of sodium chloride.

[II] The Relation between the Acquirement of the Salt-tolerance by the Osmophilic Yeasts and the Increase in Permeability of the Cell Membranes.

The growth curves of *Sacch. rouxii* in the 18% NaCl medium when they were precultured in the media containing different NaCl concentrations, were examined in the previous paper[1]. From the results, it could be said that when the yeast cells grown in a medium of a relatively low NaCl concentration such as 5% NaCl were transferred into the 18% NaCl medium, the loss of viability was remarkably slight, indicating the acquirement of a sufficent salt-tolerant property in such a low NaCl medium.

Considering the distinct increase in permeability by suspending in the 5% NaCl medium as shown in Table III and the growth curve reported in the previous paper[1], adaptive process of *Sacch. rouxii* toward the high sodium chloride concentration might be involved in some active metabolism resulting from the increase in permeability.

The cells which were suspended in the 18% NaCl medium at a low temperature, did not increase their permeability as illustrated in Table IV. When such cells were transferred to the 18% NaCl medium and incubated at 30°, a remarkable fall in viability was observed. (Table V)

When *Sacch. rouxii* cells from the ordinary culture were transferred to the concentrated sugar solution which had the same osmotic pressure as that of 18% NaCl

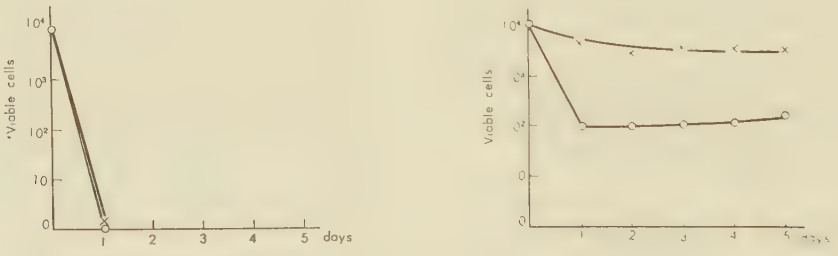

FIG. 3. Effect of Glucose on the Maintenance of Viability in the Concentrated Solution of NaCl (2).

(When the cells of the 18% NaCl culture were transferred to the saline medium)

○: NaCl 0% phosphate buffer ×: NaCl 18% phosphate buffer

TABLE V. FALL IN VIABILITY WHEN THE CELLS, WHICH WERE SUSPENDED IN THE 5% GLUCOSE 18% NaCl PHOSPHATE BUFFER AT 0°~5° FOR 3 DAYS, WERE PLATED ON THE 18% NaCl KOJI AGAR

period in suspending (days)	NaCl 0% koji agar	18% NaCl koji agar
0	10848	1920
1	5088	788
3	1200	512

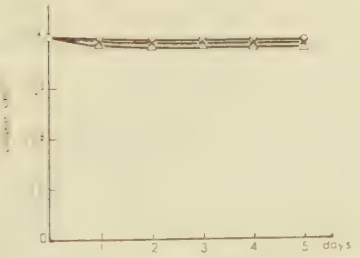

FIG. 4. The Maintenance of Viability in the Concentrated Solution af Sugar.
○: phosphate buffer
×: 50% glucose phosphate buffer
△: 40% rhamnose phosphate buffer

solution, no fall in cell viability was recognized. (Fig. 4). In this case, no change in permeability occurred as shown in Table I.

From these facts, it became much more apparent that the difference in the characteristics of salt-tolerance and sugar-tolerance of the osmophilic yeasts could be attributed to the differences in permeability of cell membranes in the concentrated solution of salts or sugars.

When the cells of *Sacch. rouxii*, precultured in the 50% glucose koji extract were plated on the agar media containing 50% gulcose and 18% NaCl, colonies of almost the same numbers were obtained in both madia. (Table VI)

It was noted that loss in viability did not occur by transferring the cells from the 50% glucose medium to the 18% NaCl medium, but any increase in permeability of the cells grown in 50% glucose medium was found not to occur as illustrated in Table I. And then, when the cells cultivated in the 50% glucose medium were transferred to the 18% NaCl medium, a similar increase in cell permeability was observed. (Table VII).

When washed cells of the ordinary culture were suspended in the 50% rhamnose phosphate buffer at 30° for 24 hours and then transferred to the 18% NaCl medium, a fall in viability was obviously observed.

Thus, the fact that yeast cells which cultivated in the 50% glucose medium can easily acquire the salt-tolerant property, may probably be explained as follows: an active metabolic process may be needed for the maintenance of the viability as a result of increased permeability of the cell membranes which occurred when the cells were transferred from the ordinary culture to the 18% NaCl medium. During growth in the 50% glucose medium, the cellular organization, by which this essential metabolic process is allowed to proceed very smoothly, may be built up. This fact was not attributable to the lack of osmotic shock when the cells were transferred between the two different media of the same osmotic preassure.

[III] Effect of Various Substances on Maintenance of Viability of the Cells in the Concentrated Solution of Sodium Chloride.

It was shown in section [1] that supply of glucose, which was available as the energy source to this yeast was essential for the maintenance of viability of the yeast cells in the saline medium. The effects of various other substances such as sugars, organic acids and amino acids were examined. The washed cells grown in the ordinary medium were suspended in the NaCl-free or 18% NaCl phosphate buffer (pH 4.8) to which the various substances had been added, and the cell supensions were incubated at 30°. The number of viable cells was determined by plating on koji agar. As shown in Fig. 5, pyruvate, acetate, succinate, citrate, *dl*-α-alanine, *l*-glutamic acid and lactose were not effective.

TABLE VI. THE SALT-TOLERANT PROPERTY OF THE CELLS OF THE GLUCOSE 50% CULTURE

strains	50% glucose koji agar	18% NaCl koji agar	strains	50% glucose koji agar	18% NaCl koji agar
1	812	720	15	1688	1800
2	1240	1128	28	1296	1336
5	1352	1232	31	1224	1296
8	1528	1512	34	792	844

Table VII. Change in Permeability when the Cells of the 50% Glucose Culture were Suspended in the 5% Glucose 18% NaCl Phosphate Buffer

period in suspending (days)	washing solution	yeast dry matter 1 g	
		K (mg)	Na (mg)
0	distilled water	6.08	0.53
1	distilled water	0	0.80
	5% glucose 18% NaCl water	1.27	—
	5% glucose 18% KCl water	—	1.67

Even under vigorous aeration, these added substances showed no effect.

DISCUSSION AND CONCLUSION

Conway et al.[3] showed that in "Na-yeast", 98% of the cellular potassium of the yeast is replaced up by the sodium and that the replaced ion could not be swept away by washing the cells with distilled water. It seems to be noteworthy that this phenomenon is merely an exchange of Na^+ and K^+ but not an increase in permeability of cell membrances. By measuring the cellular potassium and sodium contents of *Sacch. rouxii*, the present author found that permeability of cell membrances of *Sacch. rouxii* in the concentrated solution of sodium chloride is remarkably increased and consequently, almost all of cellular potassium is lost by washing with distilled water. This would be quite a different phenomenon from that of "Na-yeast."

Since permeability of cell membrances of *Sacch. rouxii*, which was cultivated in the 50% glucose medium of the same osmotic pressure as 18% NaCl remained unchanged, it became much more clear that an increase in cell permeability could not take place merely by an osmotic effect but this may be due to the effect of sodium chloride itself. Therefore, the author is of the opinion that this increase in permeability must be one of the most important points for elucidation of the mechanism of salt-tolerance.

As stated above, the cells of *Sacch. rouxii* which easily lose their cellular potassium in the saline medium, do not permit the entrance of sodium into the cell in exchange for potassium. The increase in permeability occurred by suspending the cells in the saline medium either with or without added glucose, but in the glucose-free saline medium the yeast cells rapidly die out. Moreover, when the cells which were cultivated in the 18% NaCl medium and thus fully adapted toward the high sodium chloride environment, were transferred to the glucose-free saline medium, they immediately died out. This seems to indicate that the cell the permeability of which have been changed by the action of sodium chloride can not maintain their viability unless an active metabolic process is allowed to proceed with the aid of an external substrate such as glucose.

The easy loss of cellular potassium of *Sacch. rouxii* which results from the effect of sodium chloride is of special interest since it is known that potassium plays an important role in yeast metabolism: Scott et al.[7] studied the influence of glycolytic inhibitors on the potassium and sodium contents of baker's yeast showing that glycolytic factors are involved in potassium retention. Schmidt et al.[8] observed that the absorption of orthophosphate and the formation of metaphosphate in baker's yeast cells accompanied by the absorption of cation with a strong preference for potassium. Goodman and Rothstein[9] pointed out that glycolysis and potassium ion play an important role in the uptake of phosphate by baker's yeast. Meyerhof and

7) G. T. Scott, M. A. Jacobson and M. E. Rice, *Arch. Biochem.*, **30**, 282 (1950).

8) G. Schmidt, L. Hecht and S. J. Thannhauser, *J. Biol. Chem.*, **178**, 733 (1949).

9) J. Goodman and A. Rothstein, *J. Gen. Physiol.*, **40**, 915 (1957).

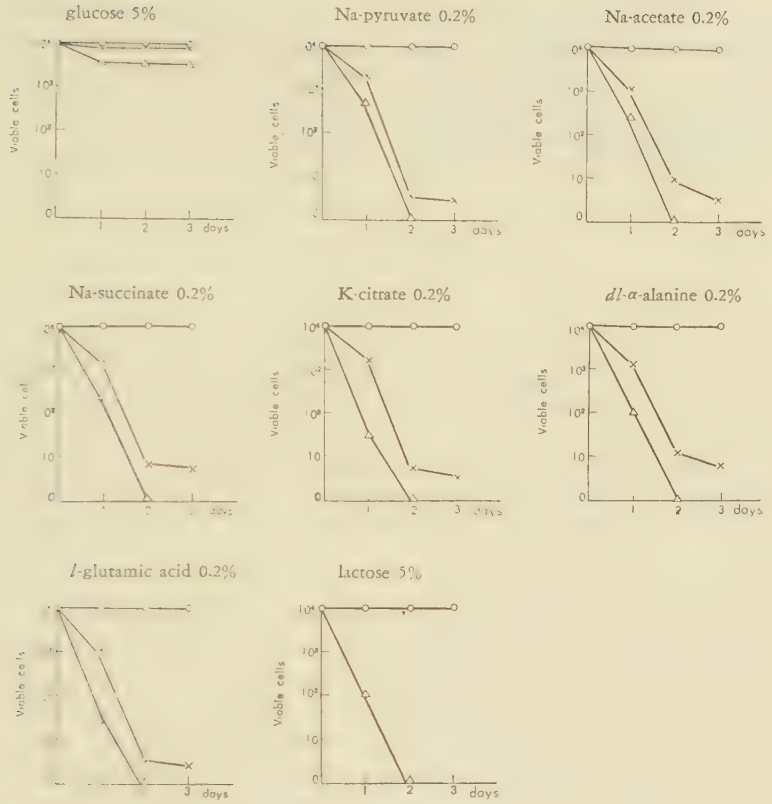

FIG. 5. Effect of Various Substances on the Maintenance of the Viability of the Cells in the Concentrated Solution of NaCl.
○: NaCl 0%, ×: NaCl 10%, △: NaCl 18%

Kaplan[10] stated that fermentation is completely absent without either K^+ or NH_4^+ and without Mg^{++}.

The effect of this tendency of easy loss of potassium on the metabolism of the salt-tolerant yeasts and its physiological significance should be further investigated in detail.

It was further noted that a change in permeability of cell membrances of *Sacch. rouxii* which was cultivated in the concentrated solution of glucose could not be observed. However, it seemed that in this case, some intracellular metabolic process different from that in the ordinary culture, may prevail supposing that this will be effective for prevention of the loss in viability when this yeast is transferred to the saline medium. In order to clarify the cause of this phenomena, intracellular metabolism of *Sacch. rouxii* in the three comparative environments, the ordinary medium, the concentrated glucose medium and the high salt medium, must be carried on further.

Acknowledgement I would like to express my sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout the course of

10) O. Meyerhof and A. Kaplan, *Arch. Biochem. and Biophys.*, **33**, 282 (1951).

this work. Great indebtness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki for their valuable suggestions. I also wish to thank Dr. R. Ishida of the Government Chemical Industrial Research Institute, Tokyo for his kind guidance in the flamephotometric analysis and Dr. M. Mogi, the Director of Noda Institute for Scientific Research for his interest and encouragement and Mr. A. Kaneko for his willing help at all times.

Studies on Osmophilic Yeasts*

Part V. Factors Affecting Growth of Soy Yeasts and Others in the Environment of a High Concentration of Sodium Chloride. (II)

By Hiroshi Ōnishi

The influences of temperature on growth and death of osmophilic yeasts in environments of both ordinary and high concentrations of sodium chloride were investigated. It was found that many osmophilic yeasts are able to grow in saline medium at a high temperature such as 40°, whereas in NaCl-free medium no growth occurred at the same temperature. Effects of various factors on this phenomena were examined. This halophilic property of yeast cells at a high temperature seemed to be due to the active metabolism of the cells in such an environment but not to the increase of heat-stability of cell proteins or enzymes that might be caused by the dehydration by effect of sodium chloride.

Specific aspects of metabolism of the osmophilic yeasts in two comparative environments of ordinary and high saline have been studied by the author[1,2]. In the previous report[1], the nutritional requirements of the osmophilic yeasts in environments of both an ordinary (NaCl-free) and a high concentration of sodium chloride (NaCl 18%) were examined and the influences of pH of the medium upon the growth in both environmental conditions were also described.

In this paper, the influences of temperature on growth and death of the osmophilic yeasts in these comparative environments were observed and it was found that maximum growth temperature of the osmophilic yeasts was increased from 32°~35° to 40° by cultivation in the saline medium. The explanation of this effect is not completely clarified, but the author may be allowed to point out one possibility here.

EXPERIMENTAL AND RESULTS

[I] Effects of Temperature on Growth of Osmophilic Yeasts in the NaCl-free and 18% NaCl Medium.

From observations on the growth of *Saccharomyces rouxii*, which played an important role in soy-brewing, in NaCl-free and 18% NaCl koji extract at various temperatures, it was found that this yeast is able to grow at 40° in the 18% NaCl medium though no growth occurred at the same temperature in the ordinary plain medium (see Fig. 2). Then, it was examined whether or not many other osmophilic yeasts show the same halophilic property at 40°. From the results given in Table I, this halophilic property at a high temperature seemed to be common in most of the osmophilic yeasts tested.

[II] Factors which support the Growth of *Saccharomyces rouxii* at a High Temperature.

(1) Effects of sodium chloride or glucose concentrations of the medium.

The minimum effective concentration of sodium chloride to support the growth of *Sacch. rouxii* at 40°, was first examined. As shown in Table II, *Sacch. rouxii* cells were able to multiply at 40° in the presence of sodium chloride at concentrations exceeding 3~4%, and similarly in the presence of glucose with a further addition of more than 15~20% to the basal medium.

(2) Effect of the conditions of preculture.

When cells of *Sacch. rouxii* grown in the 10% NaCl koji extract at 30° or 40°, were transferred to the NaCl-free koji extract, no growth occurred at 40°. Therefore, adaptation of this yeast to a higher temperature by cultivation in the high saline medium was unsuccessful.

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1) H. Ōnishi, *Bull. Agr. Chem. Soc. Japan*, **21**, 143 (1957).
2) H. Ōnishi, *Bull. Agr. Chem. Soc. Japan*, **23**, 332 (1959).

Table I. Effect of a High Concentration of Sodium Chloride on the Growth of Various Osmophilic Yeasts at the High Temperatures

(15 days cultivation in the NaCl 0% and 18% koji extract)

Strains	30°		35°		40°	
	NaCl 0%	18%	0%	18%	0%	18%
Sacch. rouxii m1	+++	++			−	++
// m2	+++	++	+++	++	−	−
// m4	+++	++			−	+
// m5	+++	+++			−	+
// m6	+++	++			−	±
// m8	+++	+++			−	+
// m9	+++	+++			−	++
// m11	+++	+++			−	+++
// s5	+++	+++			−	+++
// s7	+++	++			−	+
// s8	+++	+++	−	+++	−	−
// N5	+++	+++			−	++
// N8	+++	+++			−	++
// N28	+++	++			−	++
// A34	+++	++			−	++
S. rouxii var. *halomembranis* A31	+++	++			−	++
// sal 4	+++	++			−	+
// H5	+++	++			−	+
// j 3	+++	++			−	−
// sal 6	+++	++			−	±
S. acidifaciens s9	+++	++			−	++
S. acidifaciens var. *halomembranis* H3	+++	++			−	+
Torulopsis nodaensis N21	+++	+++	+	+++	−	−
Torulopsis halophilus N24	+++	+++	−	+++	−	−
Torulopsis versatilis F5	+++	+++	−	++	−	−
Pichia farinosa A6	+++	+++			+++	+++
Debaryomyces hansenii H70	+++	+++			+++	+++

Table II. Effects of the Concentrations of Sodium Chloride or Glucose on the Growth of *Sacch. rouxii* at 40°

Basal medium: koji extract (direct reducing sugar 10% as glucose) 40°, 10 days

Concentration of NaCl or glucose added to the basal medium	*S. rouxii* m1	*S. rouxii* m8	*S. rouxii* m11
0	−	−	−
NaCl 3%	+	−	−
NaCl 6%	++	++	++
NaCl 9%	++	++	++
glucose 5%	−	−	−
glucose 10%	−	−	−
glucose 15%	±	−	+
glucose 20%	++	++	++
glucose 30%	++	++	++

(3) Effect of vigorous aeration.

Since Tanner and Wallace[3], and also Imšenecki and Solnzeva[4] have concluded that limitation of growth in cultures of thermophilic bacteria is very probable due to exhaustion of the oxygen supply, the effect of aeration on the growth of this yeast at 40° was investigated. However, there was practically no difference in growth at 40° between the stationary culture and the shake culture; cells of *Sacch. rouxii* failed to grow in both cultures at 40° in the NaCl-free medium.

(4) Effect of addition of various substances.

As substances that prevent denaturation of proteins by heating, sodium caprylate[5] sodium dodecylsulfate[6],

3) F. W. Tanner and G. I. Wallace, *J. Bact.*, **10**, 421 (1925).
4) A. Imšenecki and L. Solnzeva, *J. Bact.*, **49**, 539 (1945).
5) P. D. Boyer et al., *J. Biol. Chem.*, **162**, 181 (1946).
6) E. L. Duggan and J. M. Luck, *J. Biol. Chem.*, **172**, 205 (1948).

Table III. Effect of Addition of Various Substances on the Growth of *Sacch. rouxii* at 40°

The Basal Medium: Bllg. 10° koji extract

Amounts of Substances added to the Basal Medium	*S. rouxii* m 1	*S. rouxii* m 8	*S. rouxii* m 11
NaBr 1 Mol	+++	++	+++
$NaNO_3$ 1 Mol	++	++	++
NaCl 1 Mol	++	++	++
KBr 1 Mol	++	++	++
KNO_3 1 Mol	++	++	++
KCl 1 Mol	+++	++	++
$MgCl_2 \cdot 6H_2O$ 0.5 Mol	++	++	++
Saccharose 10%	−(+++)	−(+++)	−(+++)
// 20%	−(+++)	(+++)	−(+++)
// 30%	++	++	++
// 40%	++	++	++
Galactose 30%	++	++	++
Glycerin 1 Mol	+	±	+
// 2 Mol	++	++	++
// 3 Mol	++	++	++
Soluble starch 2%	−(+++)	−(+++)	−(+++)
// 3%	−(+++)	−(+++)	−(+++)
Na-alginate 0.5%	−(+++)	−(+++)	−(+++)
// 1.0%	−(+++)	−(+++)	−(+++)
Agar 0.1%	−(+++)	−(+++)	−(+++)
// 0.05%	−(+++)	−(+++)	−(+++)
Peptone (Total N: 1.5%)	−(+++)	−(+++)	−(+++)
soy-bean oil 1%	−(+++)	−(+++)	−(+++)
Na-caprylate 10^{-3}Mol	−(+)	−(+)	−(+)
// 5×10^{-4}Mol	−(++)	−(+)	−(++)
Na-dodecylsulfate 3×10^{-3}Mol	−(−)	−(−)	−(−)
// 5×10^{-4}Mol	−(+++)	−(+++)	−(+++)
// 10^{-4}Mol	−(+++)	−(+++)	−(+++)
Congo red 10^{-3}Mol	−(+++)	−(+++)	−(+++)
// 10^{-4}Mol	−(+++)	−(+++)	−(+++)
// 10 Mol	−(+++)	−(+++)	−(+++)
Control (no addition)	−(+++)	−(+++)	−(+++)

( ) indicates the growth at 30°.

congo red[7] and concentrated solutions of sugars[8,9] have been reported. In addition to these substances, effects of various sodium and potassium salts, soluble starch, sugar, glycerol, sodium alginate, agar, soy-bean oil and a concentrated solution of polypeptone on growth of these yeasts at 40° were investigated.

As shown in Table III, one molar concentration of NaBr, $NaNO_3$, KNO_3 and KCl showed a similar effect as one mole of NaCl. Addition of saccharose or galactose with concentrations higher than 30% was also effective in supporting growth. However, the addition of agar (0.1%), sodium alginate (1.0%), soy-bean oil (1%), or polypeptone (1.5% as total N), was found to be ineffective in supporting growth of these yeasts at 40°.

Sodium caprylate, sodium dodecylsulfate and congo red which were generally accepted as the inhibitor of heat denaturation of proteins, were also found ineffective.

7) F. Haurowitz, Chemistry and Biology of Protein, p. 132 (1950).
8) A. C. Fay, *J. Agr. Research.*, **48**, 453 (1934).
9) T. Brosteaux and B. Erickson-Quensel, *Arch. Phys. Biol.*, **12**, 209 (1935).

Table IV. Effect of Nitrogen Sources and Vitamins on the Growth of *Sacch. rouxii* at 40°

Media	% of NaCl or glucose added to the basal medium		*S. rouxii* m 8		*S. rouxii* m 11	
			30°	40°	30°	40°
The $(NH_4)_2SO_4$ medium		0	+++	−	+++	−
	NaCl	1M	+++	++	+++	++
	//	1.5M	+++	++	+++	++
	//	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	//	15%	+++	−	+++	−
	//	20%	+++	+	+++	++
The casamino acid medium		0	+++	−	+++	−
	NaCl	1M	+++	++	+++	++
	//	1.5M	+++	++	+++	++
	//	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	//	15%	+++	++	+++	++
	//	20%	+++	++	+++	++
Thiaminie omitted medium		0	+++	−	+++	−
	NaCl	1M	+++	++	+++	++
	//	1.5M	+++	++	+++	++
	//	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	//	15%	+++	−	+++	−
	//	20%	+++	++	+++	++
Inositol omitted medium		0	+++	−	+++	−
	NaCl	1M	+++	+	+++	++
	//	1.5M	+++	++	+++	++
	//	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	//	15%	+++	−	+++	−
	//	20%	+++	++	+++	++
riboflavin, pyridoxine, niasin, and *p*-amino-benzoic acid omitted medium		0	+++	−	+++	−
	NaCl	1M	+++	++	+++	++
	//	1.5M	+++	++	+++	++
	//	2M	+++	++	+++	++
	Glucose	10%	+++	−	+++	−
	//	15%	+++	±	+++	++
	//	20%	+++	++	+++	++

(5) Effects of nitrogen sources and vitamins.

In the previous paper[1], it has been found that *Sacch. rouxii* could similarly utilize both ammonium salts and amino acids in these two contrasted environments of NaCl 0% and 18% provided the pH of medium were suitable.

Influence of the nutritional conditions on growth of these yeasts at 40° was examined.

The composition of the basal medium was as follows: glucose 5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, Vitamins (biotin 2 μg, Ca-pantothenate 400 μg, inositol 2000 μg, niacin 400 μg, *p*-aminobenzoic acid 200 μg, pyridoxine 400 μg, thiamin 400 μg, riboflavin 200 μg were added per 1 l of the medium). To 1 l of this medium, 25 cc of a buffer solution (100 g of potassium citrate and 25 g of citric acid per 1 l) was added. As nitrogen source, ammonium sulfate or vitamin free casamino acid (Difco) was added

TABLE V. INFLUENCE OF THE MINERAL REQUIREMENTS ON THE GROWTH OF *Sacch. rouxii* AT 40° (14 days)

Media	30°	40°
Basal medium	+++	−
10% NaCl basal medium	+++	++
Basal medium added with (1)* of trace elements	+++	−
Basal medium added with (2)* of trace elements	+++	−
Basal medium added with $MnSO_4 \cdot 4H_2O$ 0.04%	+++	−
Basal medium added with $FeCl_3 \cdot 6H_2O$ 0.02%	+++	−
Basal medium added with $ZnSO_4 \cdot 7H_2O$ 0.04%	+++	−
Basal medium added with $Co(NO_3)_2 \cdot 6H_2O$ 0.01%	+++	−

The basal medium: "Difco" yeast nitrogen base added with 5% glucose

* Amounts of elements per 100cc of the medium

	(1)	(2)
H_3BO_3	500 μg	5 mg
$CuSO_4$ $5H_2O$	40 μg	400 μg
KI	100 μg	1 mg
$FeCl_3$ $6H_2O$	200 μg	2 mg
$MnSO_4$ $4H_2O$	400 μg	4 mg
Na_2MoO_4 $2H_2O$	200 μg	2 mg
$ZnSO_4$ $7H_2O$	400 μg	4 mg

TABLE VI. EFFECT OF SODIUM CHLORIDE ON THE THERMAL DEATH TIME OF *Sacch. rouxii*

Strains	NaCl concentration of the media	Heating condition: 50° 10 min	50° 20 min	50° 60 min	50° 120 min	55° 1 min	55° 2 min	55° 15 min	55° 20 min
S. rouxii m 8	0%	++	−−	−−	−−	+−	−−	−−	−−
	18%	++	++	++	−−	++	++	++	−−
S. rouxii m 11	0%	++	−−	−−	−−	−−	−−	−−	−−
	18%	++	++	++	++	++	++	++	++

to the basal medium in an amount of 0.03% as total nitrogen.

Effects of vitamins were tested by employing a medium in which each vitamin was omitted one by one. In this case, biotin and Ca-pantothenate were always added because they are essential for growth of the yeasts tested.

From the results shown in Table IV, it was revealed that when either ammonium sulfate or casamino acids were employed as the nitrogen source, the yeasts were able to grow at 40°, only in the media added with NaCl (1 Mol or more) or glucose (more than 15%) but not in the plain medium, and that there were no distinct differences in the minimum effective concentration of sodium chloride to support growth of these yeasts at 40° even when each one or all of the non-essential vitamins were omitted from the basal medium.

(6) Mineral requirement.

Effects of larger amounts of the trace elements, such as B, Cu, Fe, I, Mn, Mo, Zn and Co, 10～100 times as much as those in the ordinary medium were tested.

The media employed and the results obtained are shown in Table V. The addition of these elements were all ineffective.

[III] Effect of Sodium chloride on the Thermal Death Time of *Saccharomyces rouxii*.

From the above results, it was expected that thermal death-time is elongated in the presence of sodium chloride. From the following experiment, it was confirmed that the above expectation is correct. One drop of yeast suspension was inoculated into the plain koji-extract and the 18% NaCl koji-extract in the test tubes. Attention was payed so as not to let the cell suspension come into contact with the wall of the test tubes. After

keeping the tubes for a given-time period at each temperature, they were cooled at once below 30° and incubated at 30° for 14 days. The test was carried out in duplicate (Table VI). From the results demonstrated in Table VI, it would be apparent that the thermal death-time of cells is elongated about tenfolds in the saline medium as compared with that in the plain medium.

[IV] The Growth Curve and the Death Curve of *Saccharomyces rouxii* at 30° and 40° in both the Plain and Saline Media.

Viable count of *Sacch. rouxii* cells which were incubated in the plain and 10% NaCl koji extract at 30° and 40°, was carried out. The growth curve and the death curve in each of the two media are illustrated in Fig. 1. In this example, the following seven media were employed as plating ones.: ①: koji extract agar, ②: the synthetic medium (glucose 5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, Vitamin-free casamino acid 0.4%, Yeast-extract 0.1%, Agar 2%), ③: ②+Na-acetate 0.2%, ④: ②+K-citrate 0.2%, ⑤: ②+Na-pyruvate 0.2%, ⑥: ②+DL-α-alanine 0.4%, ⑦: ②+extract of *Sacch. rouxii* cells which were cultivated in the 18% NaCl medium.

Similar growth and death curves were obtained in all of the seven plating media, showing no significant difference in the effects of these plating media on recovery from death.

[V] The Growth Curve and the Exponential Growth Rate Curve of *Saccharomyces rouxii* at Various Temperatures.

The growth curves of 2 strains of *Saccharomyces rouxii* at various temperatures in the 0% NaCl and 10% media are shown in Fig. 2. The medium employed was the same as that of [II] (5); ammonium sulfate was the nitrogen source and potassium citrate-citric acid mixture was the buffer in the medium. The extent of growth was measured turbidimetrically with a spectrophotometer. Exponential growth rates of these yeasts at various temperatures were calculated from growth curves shown in Fig. 2, and the relative values were illustrated in Fig. 3.

The exponential growth rate curve in the NaCl-free medium rapidly fell down at temperature of 32°~35° which was only 2°~3° higher than optimum growth temperature, and in the 10% NaCl medium the exponential growth rate curve did not fall down near to 40°~42.5°, though shapes of these curves in both media were similar. This seemed to explain that death of the yeast cells at higher temperatures such as 35°~40° may be caused by the inactivation of enzymes and the denaturation of proteins, and sodium chloride may apparently protect this inactivation.

[VI] Influences of the Energy Sources on Viability of *Saccharomyces rouxii* at Various Temperatures in the Plain and the Saline Medium.

Well-washed yeast cells of the fresh culture of *Sacch. rouxii* were suspended into the following four media and incubated at 10°, 30°, and 40°. Viability of the yeast cells was determined by plating on koji agar.

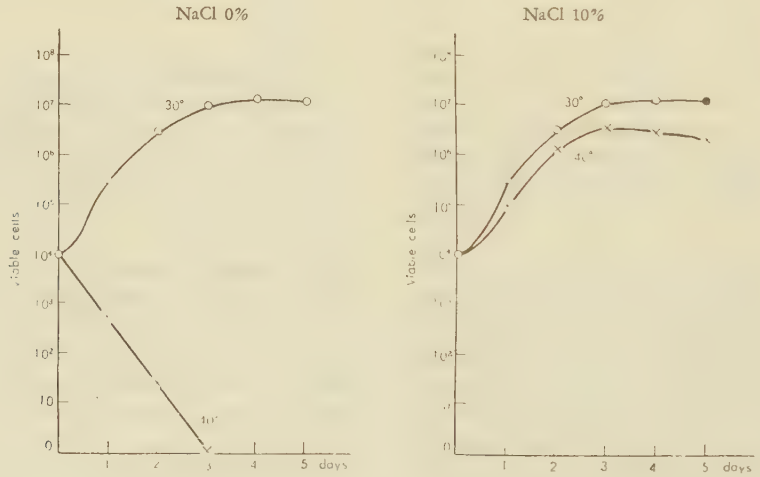

FIG. 1. The Growth Curve and the Death Curve of *Sacch. rouxii* at 30° and 40° in Both Media of the Plain and the Saline

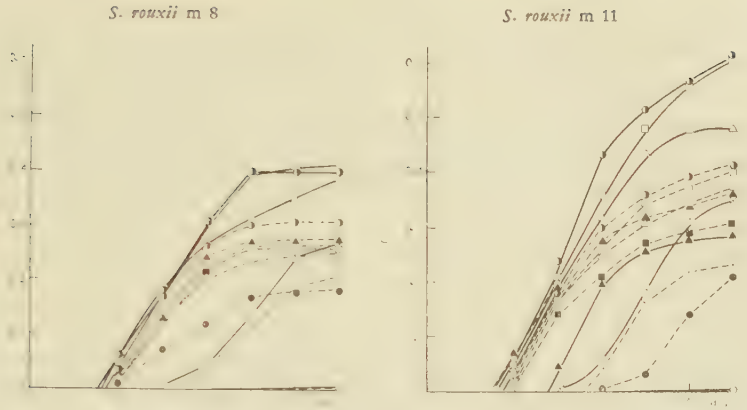

FIG. 2. The Growth Curve of *Sacch. rouxii* at Various Temperatures

○ 15° × 20° △ 25° □ 30° Solid line: NaCl 0%
◑ 32.5° ▲ 35° ■ 37.5° ● 40° Broken line: NaCl 10%

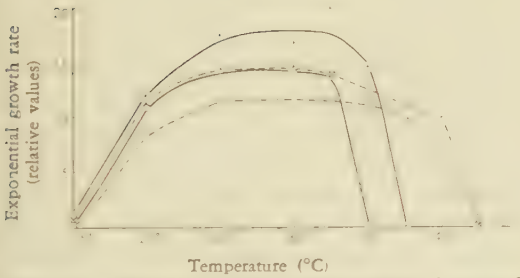

FIG. 3. The Exponential Growth Rate Curve of *Sacch. rouxii* at Various Temperatures

○: *Sacch. rouxii* m 8 Solid line: NaCl 0%
×: *Sacch. rouxii* m 11 Broken line: NaCl 10%

The suspending media employed were as follows: ①: pH 4.8 M/15 phosphate buffer, ②: NaCl 10% in pH 4.8 M/15 phosphate buffer, ③: glucose 5%, in pH 4.8 M/15 phosphate buffer, ④: NaCl 10% and glucose 5% in pH 4.8 M/15 phosphate buffer.

The results obtained are shown in Fig. 4, in which it was demonstrated that although the sole addition of 10% NaCl showed no protective effect on cell of this yeast, sodium chloride brought about a good result in maintaining viability of yeast cells in the presence of those substances such as glucose which are easily utilizable by yeasts as energy sources.

In the presence of lactose or xylose which could not

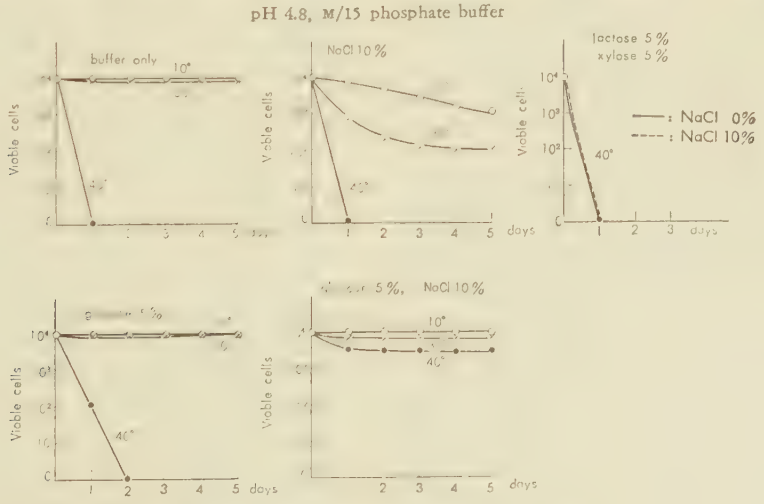

FIG. 4. Effect of the Energy Sources on Viability of *Sacch. rouxii* at Various Temperatures in the Plain and the Saline Medium

be utilized by these yeasts, the above-stated protective effect of sodium chloride was not observed.

DISCUSSION

Numerous investigations concerning the effect of salts on the heat-tolerance of microorganisms have been made but so far investigations have not reached a general agreement.

Richet, Bachrach and Cardot[10] stated that maximum growth temperature of a lactic acid bacterium is increased from 36°~37° to 41°~42° by cultivation in the presence of 3.4% KCl. van Halteren[11] has shown that the yeast is protected against the effect of heat by a concentrated solution of salts or sugars. On the other hand, Casman and Rettger[12] failed to find any protective effect of hypertonic solutions, such as 20~40% peptone, 10~15% NaCl, and 25~35% lactose on a number of organisms of the *Bac. subtilis* group. Anderson et al.[13] found that glucose and sucrose increase the heat resistance of *Bac. coagulans*, while NaCl and several organic acids have a decreasing effect.

It appears that the effect of salts is variable depending upon concentration, the kind of suspension medium and the test organism used.

The high resistance of dry proteins to heat denaturation is well known. Thus, the increased thermal resistance of cells or spores of microorganisms in the concentrated solutions has hitherto principally been attributed to difficulty of heat denaturation of enzyme proteins because of the actual dehydration of cell contents in solutions of high osmotic pressure[8,14].

As shown in Figs. 2 and 3, it is probable that the failure of *Sacch. rouxii* to grow in an ordinary medium at a higher temperature such as 35° to 40° is due to the inactivation of enzymes or denaturation of proteinous molecules in the cells and that in the saline medium sodium chloride apparenlty acts to protect the protein molecules from heat denaturation. However, it was demonstrated in Fig. 4 that *Sacch. rouxii* cells rapidly die out in a pH 4.8 M/15 phosphate buffer without glucose in spite of the presence of 10% NaCl, and only in the concurrent presence of easily utilizable energy sources, the protective effect of sodium chloride was recognized. In the previous paper[2], it was reported that *Sacch. rouxii*, a typical salt-tolerant species, rapidly dies out in the presence of sodium chloride if the medium is devoid of the easily utilizable energy sources, and that active metabolism is indispensable for maintaining viability in a high saline medium, in which the permeability of cell membrane was significantly changed. These facts together with the present experimental data seem to indicate that the halophilic property of the osmophilic yeasts at higher temperatures is maintained by the active metabolism of utilizing external substrates rather than by merely inhibiting denaturation of enzyme proteins due to dehydration by sodium chloride. This supposition may also be supported by the fact that this halophilic phenomena at higher temperatures occurs in a saline environment as low as 3~6% NaCl, which seems to be ineffective to cause a remarkable dehydration of cells of these osmophilic yeasts.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki and Prof. A. Takamiya and Dr. M. Amaha for their valuable suggestions. Thanks are also due to Dr. M. Mogi, the Director of Noda Institute for Scientific Research for his encouragement in the cource of this work.

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Studies on Osmophilic Yeasts*

Part VI. Glycerol Production by the Salt-tolerant Yeasts in the Medium with High Concentration of Sodium Chloride

By Hiroshi Ōnishi

Saccharomyces rouxii which is a typical salt-tolerant yeast and plays an important role in soy-brewing, was found to ferment glucose giving high yields of glycerol in a high saline meduim under aerobic conditions, though only a small amount of glycerol was produced in the ordinary medium. An amount as much as 40~50% of glucose fermented was converted to glycerol under the above conditions.

In the previous reports[1,2], the author has conducted comparative studies on the physiological characteristics of salt-tolerant yeasts in two contrasting conditions; one, in the ordinary NaCl-free medium and the other in the 18% NaCl medium. After examining the material balances for fermentation in the NaCl-free and the 18% NaCl medium by the salt-tolerant yeasts of various genera and species[3,4,5] which were isolated from soy-mashes, the present author has found that *Saccharomyces rouxii* gives high yields of glycerol in the 18% NaCl medium under aerobic conditions. This paper deals with the effects of various fermentation conditions on glycerol production in a high saline medium.

MATERIALS AND METHOD

Yeast strains employed: 29 strains of *Sacch. rouxii*, isolated from soy-mashes and miso-pastes and showed high salt-tolerance, were employed.

Media and conditions of fermentation: The basal medium composition was follows: glucose 10%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, vitamin-free casamino acid (Difco) 0.4%, Yeast extract (Difco) 0.1%. Various modifications of this medium were made by using different concentrations of salts and by adding various kinds of nitrogen sources. In the stationary and anaerobic cultures, 100 ml of these media were placed in 150 ml Erlenmyer flasks, sterilized, inoculated and incubated at 30°. In the aerobic culture, 80 ml of these media were placed in 500 ml shaking flasks, and the flasks were shaken on a reciprocal shaker operating at 120 r.p.m. with a stroke of 75 mm at 30°. After 10 days' incubations, the fermentation products were analysed.

Analytical method:

Glucose was determined by the Fehling-Lehmann-Schoorl method[6].

Determination of glycerol was conducted by the method of Neish[7]. The amount of crude glycerol, in which other polyhydric alcohols such as mannitol and erythritol were contaminated, was determined by oxidation with periodic acid followed by colorimetric determination of formaldehyde formed with a chromotropic acid reagent. The values obtained were corrected for glucose presented by running a standard glucose sample in parallel with the fermentation solutions. It was confirmed that the method of Neish is fully applicable to the fermentation solution of 18% NaCl without any modification. Pure glycerol was separated on water-celite columns using a mixture of 25% benzene and

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Table I. Glycerol Production by *Sacch. rouxii* in the NaCl-free and 18% NaCl Media under Aerobic and Anaerobic Conditions

Cultural conditions	Strains	The NaCl-free medium: % of glucose consumed	% of crude glycerol formed	yield of glycerol on glucose consumed	The 18% NaCl medium: % of glucose consumed	% of crude glycerol formed	yield of glycerol on glucose consumed
Anaerobic*	N 28	6.12	0.457	7.4%	6.05	0.511	8.4%
	E 7	6.54	0.495	7.5	5.86	0.614	10.5
Stationary*	N 28	5.24	0.409	7.8	5.56	0.573	10.3
	E 7	6.20	0.504	8.1	5.10	0.759	14.8
Shaking	N 28	9.02	1.289	14.2	7.66	2.843	37.1
	E 7	9.18	1.995	21.7	7.83	2.248	28.7

Initial glucose concentration is 9.51%.
* These cultures were analysed after 15~20 days' incubation at 30°.

Table II. Glycerol Production by Various Strains of *Sacch. rouxii* in the 18% NaCl Medium with Aeration

Strains	% of glucose consumed	% of crude glycerol produced	yield of glycerol on glucose consumed	Strains	% of glucose consumed	% of crude glycerol produced	yield of glycerol on glucose consumed
m 1	6.39	2.182	34.1%	R 5	3.03	0.761	25.1%
m 2	2.10	0.503	24.0	R 6	6.53	2.256	34.5
m 3	8.25	3.588	43.4	N 5	6.07	2.062	34.0
m 4	3.44	0.983	28.6	N 8	6.26	2.013	32.1
m 11	3.24	0.837	25.8	N 16	1.29	0.374	28.9
s 2	2.83	0.765	27.0	N 22	3.37	0.841	25.0
s 5	7.52	2.616	34.8	N 28	6.63	2.399	36.1
s 7	2.83	0.825	29.2	A 31	6.86	1.816	26.5
s 8	6.73	1.902	28.2	A 34	3.86	0.936	24.2
s 9	4.92	1.758	35.7	H 1	1.56	0.487	31.2
sal 1	1.84	0.415	22.6	H 4	1.49	0.405	27.2
sal 2	4.05	0.942	23.3	H 5	1.42	0.418	29.4
sal 4	1.15	0.310	26.9	H 7	5.75	1.348	23.4
j 3	5.43	1.663	30.6	E 7	8.22	2.537	30.8
R 4	2.83	0.755	26.6				

Initial glucose concentration is 10.0%.

75% *n*-butanol saturated with water as a developing solvent[7] and analysed by the above procedure of periodic oxidation.

Paper chromatograms were developed with a solvent composed of *n*-butanol, ethanol and water (4 : 1 : 5 by volume) according to the method of Hackman[8], and glycerol and similar compounds were detected by the method of Yoda[9]. The dried chromatogram was first sprayed with 0.5% aqueous KIO_4. After 1~2 minutes, the paper was sprayed with the following reagents: 15% aqueous $MnSO_4$: saturated *p*-*p*′-tetramethyl diaminodiphenylmethane in 2N acetic acid=1 : 1 v/v. White spots on the blue background indicate the locations of α-glycols.

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EXPERIMENTAL AND RESULTS

(1) Effect of Aeration.

Glycerol production by two strains of *Sacch. rouxii* when they were cultured aerobically and anaerobically

in two media i.e. NaCl-free and 18% NaCl was examined. The results are shown in Table I. In the NaCl-free medium, the yield of glycerol in the anaerobic culture was 7~8% on glucose consumed, and this yield was increased up to 14~20% with aeration. In the 18% NaCl medium, as much as 30~40% of glucose consumed was converted to glycerol in the aerobic conditions.

(2) Selection of an Excellent Strain for Glycerol Production.

In order to select the excellent strain, the glycerol production in the 18% NaCl medium by 29 strains of *Sacch. rouxii* which were isolated from soy-mashes and miso-pastes, was examined. As shown in Table II, the rate of fermentation and the yield of glycerol varied with the strains but in general, a high glycerol yield ranging from 22.6~43.4% on sugar consumed was obtained by all the strains tested. Among these, the strain m 3 was found to be the most excellent strain for this production.

(3) Effects of Nitrogen Sources and Concentrations of Salts.

Employing strain m 3, the effects of various nitrogen sources and the concentration of NaCl or KCl on the glycerol production were examined. As nitrogen source, each of ammonium sulfate, ammonium chloride, urea, polypeptone, beef extract, ammonium lactate and ammonium acetate was added to the basal medium in place of casamino acid. The amounts of these nitrogen sources added are shown in Table III. In the media containing $(NH_4)_2SO_4$ or NH_4Cl, K-citrate-citric acid buffer was also added according to the reason previously reported[1]. Although the rate of fermentation differed in each medium with different nitrogen sources, very high yield of glycerol such as from 43~50% on glucose consumed was obtained in all cases. The yield of glycerol was remarkably increased with increasing concentration of NaCl or KCl (Table III).

(4) Paper Chromatogram of the Fermentation Broth.

In the determination of glycerol, 2–3 butanediol and acetoin did not interfere, and the interfering effect of glucose was eliminated by correction. However, other polyhydric alcohols such as mannitol and erythritol would interfere to a great extent if they were present. Detection of other polyhydric alcohols in the fermentation broth of 18% NaCl (Table II) was carried out by means of paper chromatography. The result are illustrated in Fig. 1 and Photograph 1. These indicate that glycerol is the main product by aerobic fermentation in a high saline medium. Other polyhydric alcohols could not be obviously detected.

(5) Determination of True Glycerol by Partition Chromatography.

It was first examined whether or not partition chromatography by Neish[7] is applicable to the high saline solution. Solutions of 18% NaCl containing glycerol, mannitol, glucose, ethanediol and/or 1–2 propanediol, were prepared and diluted to a suitable concentration and then analyzed by partition chromatography after Neish[7]. As shown in Table IV, glycerol, sugar alcohol,

Table III. Effects of Various Nitrogen Sources and the Concentrations of Sodium Chloride and Potassium Chloride

Media	% of glucose consumed	% of crude glycerol produced	yield of glycerol on glucose consumed
Basal medium	8.72	0.929	10.6%
〃 〃 +NaCl 6%	8.79	2.580	29.3
〃 〃 +NaCl 12%	8.57	2.195	25.6
〃 〃 +NaCl 18%	8.43	3.630	43.1
〃 〃 +KCl 11%	8.72	2.970	34.0
〃 〃 +KCl 22%	8.65	3.765	43.5
〃 〃 +NaCl 18%+Polypeptone 1%	8.39	3.018	36.0
〃 〃 + 〃 〃 +$(NH_4)_2SO_4$ 0.2%	6.85	3.252	47.4
〃 〃 + 〃 〃 +NH_4Cl 0.2%	3.48	1.746	50.1
〃 〃 + 〃 〃 +Urea 0.02%	4.99	2.533	50.7
〃 〃 + 〃 〃 +Ammonium lactate 0.2%	6.83	3.248	47.5
〃 〃 + 〃 〃 +Ammonium acetate 0.2%	6.55	3.229	49.2
〃 〃 + 〃 〃 +Beef extract 0.5%	8.20	3.589	43.8

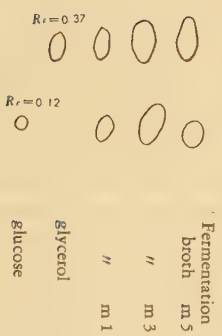
Initial glucose concentration is 10.65%.

TABLE IV. DETERMINATION OF GLYCEROL BY PARTITION CHROMATOGRAPHY

Solutions of glycerol, α-glycols, sugar, and fermentation broth	Values before developing*	Values after developing* Fractions			
		I	II	III	IV
glycerol	4.37 mg	0	0	0	4.37 mg
mannitol	3.92 mg	0	0	0	0
glucose	0.20 mg	0	0	0	0
mixture of glycerol, mannitol and glucose	4.22 mg	0	0	0	3.12 mg
ethanediol	3.01 mg	0	0	3.01 mg	0
1–2 propanediol	2.90 mg	0	0	3.00 mg	0
18% NaCl fermentation broth	3.85 mg	0	0	0	3.25 mg

* This values are all indicated as glycerol.

FIG. 1. Paper chromatogram of the fermented broth.



Photograph 1. Paper chromatogram of the fermented broth.

sugar, 1–2 propanediol and ethanediol were separated each other with satisfactory recovery. From the results of true glycerol determination in the 18% NaCl fermentation broth by this partition chromatography, 85% of the crude glycerol determined was found to be true glycerol. The detection of other compounds such as sugar alcohols which might be formed together with glycerol, and the results will be reported in the following paper.

DISCUSSIONS AND CONCLUSION

It is well-known that yeasts produce large quantities of glycerol in the presence of sulfite[10] or alkali[11]. However, fermentation processes without the addition of these agents may be commercially adventageous. Therefore, glycerol production by a normal fermentation process has recently been investigated by several workers: Nickerson and Carrol[12] found that *Zygosaccharomyces acidifaciens* produces glycerol in a yield of about 20% on the weight of sugar fermented in a normal fermentation process. Neish, Ledingham and Blackwood[13] showed that another type of fermentation by *Bacillus licheniformis* leads to glycerol production together with 2–3 butanediol, lactic acid, ethanol and formic acid. Spencer and Sallans[14] reported that osmophilic yeasts isolated from fermenting honey and other sources produce one or more of the sugar alcohols, as glycerol, erythritol, D-arabitol, mannitol and two unidentified polyhydric alcohols. As much as 60% of the glucose dissimilated was converted to polyhydric alcohols. Fukui and Mohara[15] studied on the formation of glycerol by the use of sulfathiazole as an antagonist against yeast cocarboxylase. For example, when 0.8 g of sulfathiazole was added to 100 ml of the medium and the medium was fermented by 2 g of pressed yeast, 3.4 g of glycerol and 2.6 g of ethylalcohol were formed from 10 g of glucose after 7 days at 30°.

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In the present paper, it has been reported that yeasts produce much higher yield of glycerol (as much as 40~50% on sugar fermented) in a high saline medium under aerobic conditition, although a small amount is formed in the ordinary plain medium. This fact is of special interest not only that sodium chloride affects the yeast metabolism to alter the fermentation pathway but also that the industrial process of glycerol production is proved to be promising in view of its high yield and simplicity in procedure as compard with those by the use of steering agents.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki for their valuable suggestions. Thanks are also due to Dr. M. Mogi, the Director of Noda Institute for Scientific Research, for his encouragement and to Mr. A. Kaneko for his willing help at all stages of this work.

Studies on the Proteolytic Enzymes of Black Aspergilli*

Part V. The Specificity of Crystalline *Asp. Saitoi* Proteinase by Application of the Sanger DNP-Method

By Fumihiko Yoshida, Michitaro Nagasawa and Eiji Ichishima

The specificity of crystalline *Asp. Saitoi* proteinase on oxidized lysozyme has been investigated by application of the Sanger DNP-method.

It was found that this proteinase has a much broader specificity as compared with pepsin and *Bac. subtilis* proteinase.

Details of the specificity of well-known proteolytic enzymes have been investigated employing synthetic peptides and their derivatives[1].

However, at present, it is also possible to use proteins as a substrate for this purpose adopting suitable techniques for end-group analysis studies in peptides and proteins according to the Sanger DNP-method[2,3,4]. For this reason, horse globin and ovalbumin[5] have been used for the determination of specificity of certain proteolytic enzymes.

In the previous paper of this series, the specificity of *Asp. Saitoi* proteinase on synthetic substrates was investigated, and it was found that the enzyme had much broader specificity than pepsin[6]. In this paper, a study has been conducted to determine the specificity of this enzyme through analysis of free amino acids and N-terminal amino acid residues of peptides liberated from oxidized lysozyme by the enzymatic digestion. The abbreviation for amino acid residues as suggested by Brand and Edsall[7] are adopted here.

EXPERIMENTAL

1) Standard DNP-amino acids: Standard DNP-amino acids were supplied by the Wakō Pure Chemical Co., Ltd. For the paper chromatographic analysis, the ether soluble DNP-amino acids were dissolved in acetone to M/100 solutions, and the water soluble ones were dissolved in methylethylketone containing a small amount of hydrochloric acid.

2) Paper chromatography: The chromatagraphic separation of DNP-amino acids was effected two dimensionally on a buffered filter paper (40×40 cm), Tōyō Roshi No. 51, which had been previously sprayed with M/10 phthalate buffer (pH 6.0) and dried at room temperature. Many of the solvent systems tried proved to be unsuitable, because the DNP-amino acids ran fast near the solvent front or else somewhat 'tailed'. The solvent systems which can be successfully used are as follows:

Ether soluble fraction

1. tert-AmOH-phthalate buffer (pH 6.0)[8]
2. 1.5 M phosphate buffer (pH 6.0)[9]

Water soluble fraction

1. *n*-BuOH-1% NH_3 (1/1, v/v)[10]
2. 0.75 M phosphate buffer (pH 6.0)[11]

3) Crystalline *Asp. Saitoi* proteinase: A crystalline *Asp. Saitoi* proteinase was prepared according to

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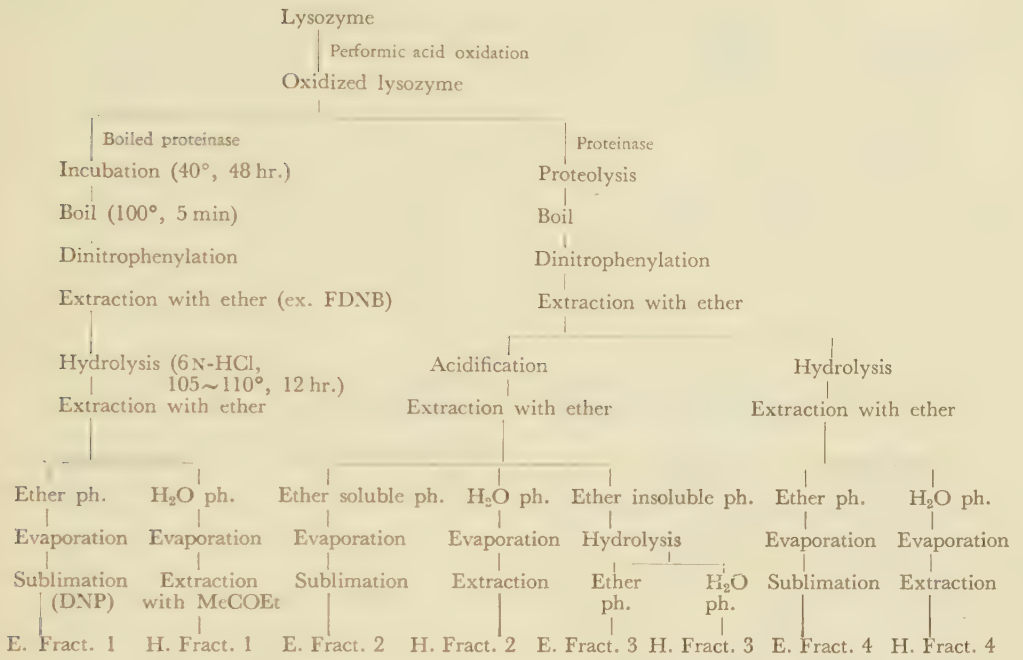


FIG. I. Procedure for the Determination of N-Terminal Residues

the method of Yoshida[12].

4) Substrate (Oxidation of lysozyme with performic acid[13]): Five ml of 30% hydrogen peroxide (R.G.) was added to 45 ml of 90% formic acid and after standing at room temperature for 1 hr., the mixture was cooled down to −8° in a freezer. The performic acid mixture was added to a cold solution of 1 gr of lysozyme (supplied by N.B.C.) in 10 ml of 90% formic acid. After being left for 30 min. at −8° (or at the same temperature) the reaction mixture was diluted with 900 ml of ice water, the diluted mixture immediately frozen with ethanol-dry ice and the solvents were removed by lyophilization. The residue was dissolved in 50 ml of water and lyophilized again.

5) Enzymatic hydrolysis of substrate: Thirty mg. of oxidized lysozyme dissolved in 3 ml of dilute hydrochloric acid solution (pH 2.7) was mixed with 0.5 ml of enzyme solution containing 0.3 mg of *Asp. Saitoi* proteinase and allowed to react at 40° for 48 hours. After boiling to inactivate the enzyme, the solution was lyophilized. As a control experiment, 3 ml of oxidized lysozyme solutuion was mixed with 0.5 ml of the previously boiled enzyme solution and the mixture was treated as above.

6) Reaction with 2,4-dinitrofluorobenzene: Since it was recognized that the enzyme splitted protein molecules to samller fragments than pepsin[14], the scheme shown in Fig. 1 has been used to determine free amino acids and N-terminal residues in hydrolyzate. In order to identify the N-terminal residues of both undigested proteins and digested products, the method of Okunuki et al.[15] was applied.

Thirty mg of hydrolyzate was dissolved in 3 ml of water, and 30 mg of sodium bicarbonate was added; the solution was adjusted to pH 8.5 by 0.8 M sodium bicarbonate buffer. Then, the buffered solution was mixed with 10 ml of alcoholic solution of 2,4-dinitrofluorobenzen (DNFB) (0.1 ml DNFB for the control, 0.5 ml DNFB for 48 hour's digestant) and the resulting mixture was shaken for 3 hr. at room temperature. After removing ethanol in vacuo, 5 ml of water was added and the excess DNFB was extracted with ether. Ether was removed from the aqueous layer in vacuo

12) F. Yoshida and M. Nagasawa, *Bull. Agr. Chem. Soc. Japan*, **22**, 32 (1958).
13) G. Toennis and R. P. Homiller, *J. Am. Chem. Soc.*, **64**, 3054 (1942).
14) F. Yoshida and M. Nagasawa, *Proc. Intern. Symposium Enzyme Chem.*, **2**, 504 (1957).
15) K. Okunuki et al., *J. Biochem. Japan.*, **43**, 857 (1956).

TABLE I. RECOVERY OF STANDARD DNP-AMINO ACIDS ON PAPERCHROMATOGRAPHY (2-Dimensional)

DNP-Amino Acids	Photometric Solvents	Recovery (%)	DNP-Amino Acids	Photometric Solvents	Recovery (%)
Ala	1%-$NaHCO_3$	61	Thr	1%-$NaHCO_3$	76
Asp+Glu	//	73	di-Tyr	//	87
Gly	//	84	Try	//	80
Leu+Ileu	//	84	Val	//	85
di-Lys	Glacial AcOH	94			
Met	1%-$NaHCO_3$	69	α-Arg	1 N-HCl	87
Phe	//	67	$CySO_3H$	//	74
Pro	//	70	di-His	//	83
Ser	//	89	ε-Lys	//	91

TABLE II. N-TERMINAL RESIDUES IN DIGESTION PRODUCT FROM OXIDIZED LYSOZYME BY THE ACTION OF *Asp. Saitoi* PROTEINASE

DNP-Amino Acids	Moles of residue per molecular of oxidized lysozyme*	Moles of residues detected as N-Terminal**				
		Fract. 1 (Control)		Fract. 2	Fract. 3	Fract. 4
Ala	10−12			0.76	0.47	3.21
Asp+Glu	20+5	0.32		2.28	0.47	6.18
Gly	11−12		Gly..1	0.66		3.18
			..2	0.65		
			..3	0.43		
di-Lys	1 (+5)	0.465		1.06	0.49	0.75
Leu+Ileu	8+6		Leu+..1	1.70		4.10
			Ileu..2	1.54		
Phe	3			0.26		1.18
Pro	2			0.84		0.78
Ser	9	0.05	Ser..1	0.33	0.19	1.19
			..2	0.18		
Thr	7			0.21		1.71
di-Tyr	3			0.67	0.34	0.67
Val	5−6	0.07		0.39	0.33	0.99
Unknown 1.E				2.44		0.40
2.E						0.86
α-Arg	11					1.67
$CySO_3H$	10			3.06	1.37	3.92
di-His	1					0.66
ε-Lys	(Initial 5)	2.90		1.50	1.57	3.16
Unknown 1.H		+		0.14		0.17
2.H				0.26	0.29	0.19
3.H				0.87	0.44	0.26
4.H				0.44		
5.H				0.30		
6.H				0.58		
7.H				0.18		0.57
8.H				0.19		+
9.H						0.67

* Amino acids composition of oxidized lysozyme was assumed according to Thompson.[17]

** Recovery factor of hydrolysis were adopted according to the literature.[2,18,19]

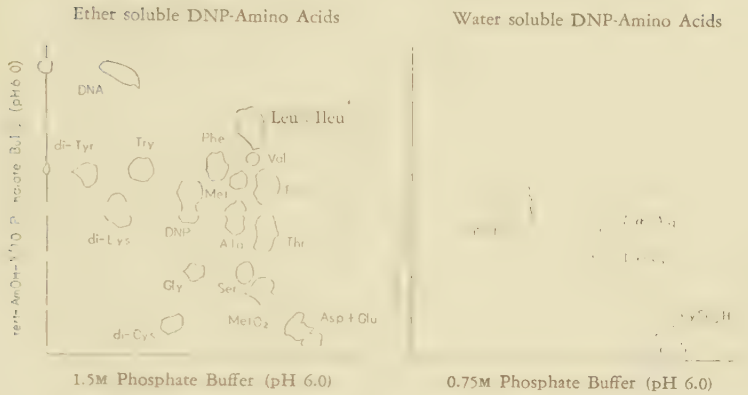


FIG. 2. Paperchromatogram of Standard DNP-Amino Acids

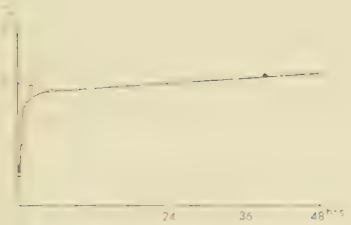


FIG. 3. Digestion Curve of Oxidized Lysozyme by *Asp. Saitoi* Proteinase

and the solution was neutralizated with a few drops of conc. hydrochloric acid. After the addition of an equal volume of conc. hydrochloric acid to the solution, the mixture was hydrolyzed in a sealed tube at 115~120° for 12 hr.

On the other hand, the samples from which the excess DNFB and DNA (dinitroaniline) were removed were acidified with conc. hydrochloric acid and extracted with ether to determine free amino acids and their derivatives. DNP-amino acids were extracted with ether and most of dinitrophenol (DNP) was removed according to the method of Mills[16]. The aqueous phase was then evaporated in vacuo and extracted with methylethylketone.

7) Quantitative analysis[9]: DNP-amino acids were separated with paper chromatography using the solvents mentioned previously. Each spot was eluted with suitable solvent systems and the amount of each DNP-amino acid was determined spectrophotometrically at 360 mμ (for most of DNP-amino acids), 355 mμ (for di-DNP-Lys), 380 mμ (for DNP-Pro), respectively.

16) G. L. Mills, *Biochem. J.*, **50**, 707 (1952).

RESULTS AND DISCUSSION

In general, the separation of DNP-amino acids on paper chromatogram has resulted in only a limited success due to the 'tailing' of spots. Blackburn et al.[8] observed that DNP-amino acids might be successfully separated on buffered one-dimensional paper chromatograms, compact spots without 'tailing' which possess characteristic rates (R_F value) being formed in suitable solvents. Levy[9] established a method for the quantitative microanalysis of DNP-amino acid mixture on a two-dimensional filter paper chromatogram, which is followed by quantitative elution from the paper and estimation at 360 mμ.

The standard DNP-amino acids chromatogram and their recoveries on the paper chromatogram are shown in Fig. 2 and Table I, respectively.

The course of hydrolysis of oxidized lysozyme by *Asp. Saitoi* proteinase is shown in Fig. 3.

The results of analysis by the Sanger DNP-method are summarized in Fig. 4-A to 4-D and Table II. As shown in Fig. 4-A very small quantities of DNP-derivatives of Val, Ser, and Asp+Glu appearing on the chromatogram may represent impurities in the preparation of terminal groups resulting from partial breakdown of peptide chain of the substrate by performic acid oxidation. From Fig. 4-B and Fig. 4-D, it was recognized that Ala, Asp +Glu, Gly, Leu+Ileu,

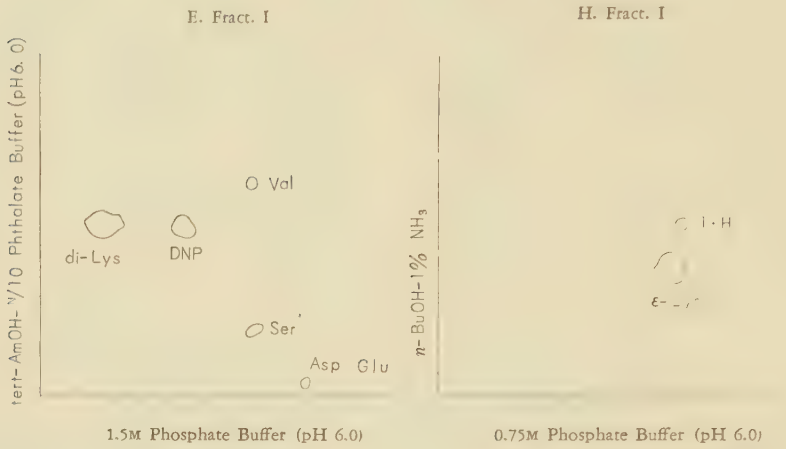


FIG. 4–A. Paperchromatogram of N-Terminal Residues in Digestion Product from Oxidized Lysozyme by the Action of *Asp. Saitoi* Proteinase

The abbreviation of E. and H. indicated unknown DNP-derivatives in the ether soluble phase, water soluble phase respectively.

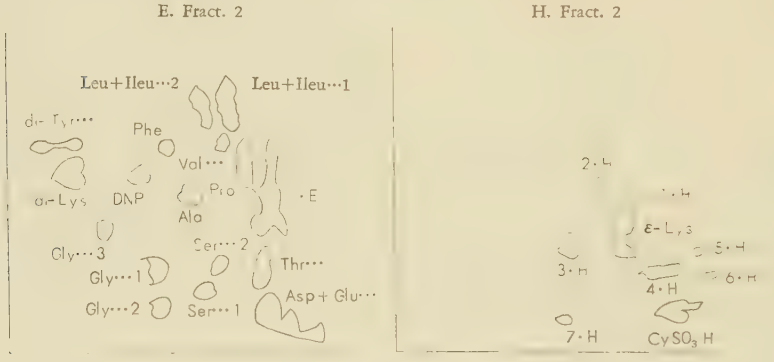


FIG. 4–B

The abbreviation Thr..., etc. indicated the DNP-peptide, which corresponded to DNP-Thr on paperchromatogram.

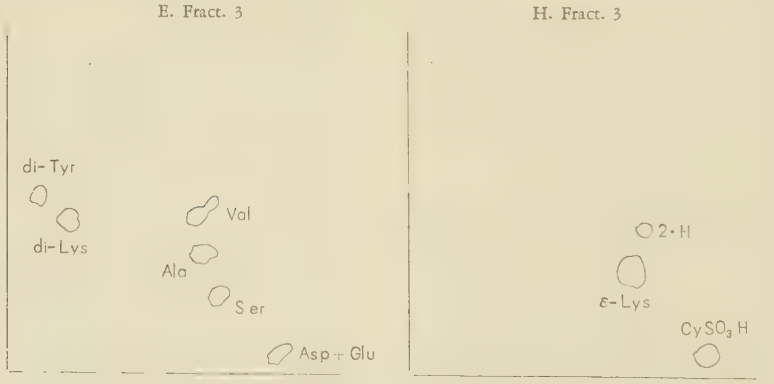


FIG. 4–C

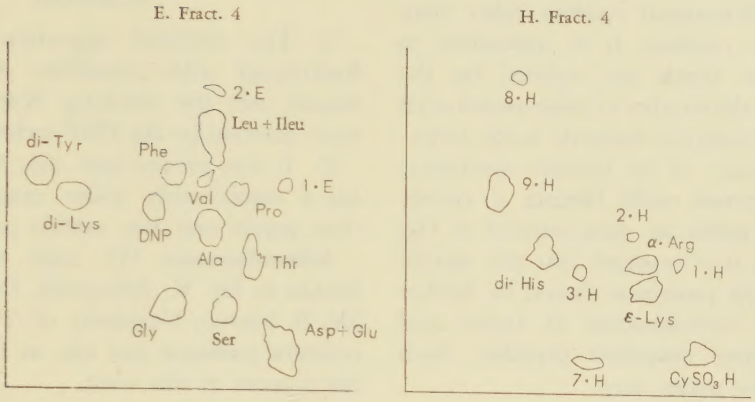
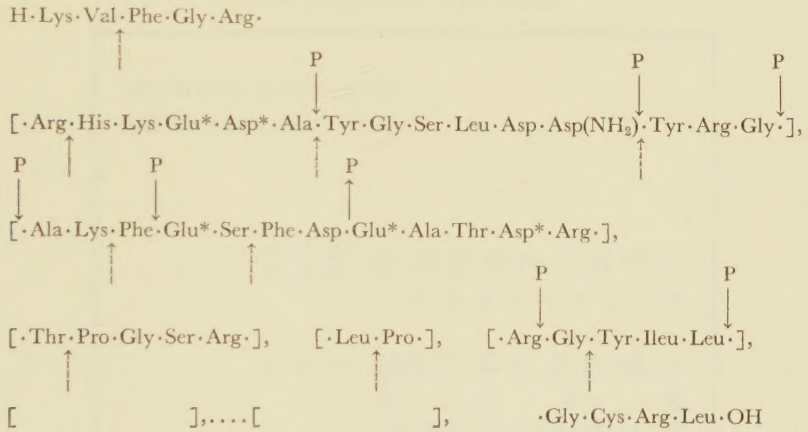


FIG. 4–D

TABLE III. SITE OF CLEAVAGE OF OXIDIZED LYSOZYME BY *Asp. Saitoi* PROTEINASE



⟵ Bonds split by *Asp. Saitoi* Proteinase.
⟵ Some of these bonds split by *Asp. Saitoi* proteinase.
⟵P Bonds split by pepsin.
* It has not been determined whether or not these residues contain an amide group.

Lys, Phe, Pro, Ser, Thr, Tyr, Val, Arg, $CySO_3H$, and His are found as N-terminal residues of peptides resulting from oxidized lysozyme by the action of *Asp. Saitoi* proteinase. In comparison with N-terminal residues in the digestion products with pepsin[20] and *Bac. subtilis* proteinase[15], the mold proteinase appears to have a considerably wider range of specificity than the other two enzymes.

In recent years, though the structure of lysozyme has been studied by Thompson[17,21], Fromageot and Jolles[22], and others, some amino acid sequences are still obscure.

The main site of lysozyme molecule at which splitting occurrs under the action of *Asp. Saitoi* proteinase and pepsin, in concern of His, Phe,

17) A. R. Thompson, *Biochem. J.*, **60**, 507 (1955).
18) W. A. Schroeder, *J. Am. Chem. Soc.*, **75**, 4612 (1952).
19) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).
20) P. Jolles et al., *Biochim. Biophys. Acta.*, **27**, 439 (1958).
21) A. R. Thompson, *Biochem. J.*, **61**, 253 (1955).
22) C. Fromageot and P. Jolles, *Proc. Intern. Symposium Enzyme Chem.*, **2**, 504 (1957).

Pro and Tyr residues are indicated in Table III. Regarding the N-terminal residues other than the above four residues, it is impossible to determine which bonds are splitted by the enzyme, because the number of these amino acids contained in a lysozyme molecule is too large.

However, because of its broader specificity, *Asp. Saitoi* proteinase could liberate a variety of unsplitted peptides as demonstrated in Fig. 4-B. Therefore, it is expected that the specificity of *Asp. Saitoi* proteinase should be further clarified by the determination of amino acid sequences of these unsplitted peptides. Such attempts are now under way.

SUMMARY

1. The oxidized egg-white lysozyme was hydrolyzed with crystalline *Asp. Saitoi* proteinase and the resulting N-terminal residues were detected by the DNP-technique of Sanger.

2. It was proved that *Asp. Saitoi* proteinase has a considerably wider range of specificity than pepsin and *Bac. subtilis* proteinase.

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